GAP-43 mRNA in Growth Cones Is Associated with HuD and Ribosomes

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ABSTRACT: The neuron-specific ELAV/Hu family member, HuD, interacts with and stabilizes GAP-43 mRNA in developing neurons, and leads to increased levels of GAP-43 protein. As GAP-43 protein is enriched in growth cones, it is of interest to determine if HuD and GAP-43 mRNA are associated in developing growth cones. HuD granules in growth cones are found in the central domain that is rich in microtubules and ribosomes, in the peripheral domain with its actin network, and in filopodia. This distribution of HuD granules in growth cones is dependent on actin filaments but not on microtubules. GAP-43 mRNA is localized in granules found in both the central and peripheral domains, but not in filopodia. Ribosomes were extensively colocalized with HuD and GAP-43 mRNA granules in the central domain, consistent with a role in the control of GAP-43 mRNA stability in the growth cone. Together, these results demonstrate that many of the components necessary for GAP-43 mRNA translation/stabilization are present within growth cones. © 2004 Wiley Periodicals, Inc. J Neurobiol 61: 222–235, 2004

Keywords: **GAP-43; HuD; growth cone; mRNA; ELAV/Hu family; immunocytochemistry; *in situ* hybridization**

INTRODUCTION

The metabolic and structural polarity of nerve cells is generated during development and is crucial for their function. Targeting of developmentally regulated proteins to elongating axons, dendrites, and growth cones is necessary to establish this polarity. Developmentally regulated proteins are selectively distributed to the elongating axons or dendrites (Goslin et al., 1988; Skene and Willard, 1981a; Stein et al., 1988). One mechanism for the differential distribution of proteins in neurons is the transport of proteins from the cell body to growth cones. Another mechanism is the local translation at the growth cone (Bassell et al., 1998; Brittis et al., 2002; Campbell and Holt, 2001; Crino and Eberwine, 1996; Ming et al., 2002). In this latter case, transport of mRNAs and molecules used in translation is required for specific localization of pro-

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teins (Lipshitz and Smibert, 2000). There is evidence that mRNAs bound to proteins are transported in ribonucleic protein (RNP) granules from the neuronal cell body to dendrites and dendritic growth cones (Giuditta et al., 2002; Kiebler and DesGroseillers, 2000; Krichevsky and Kosik, 2001). There is also evidence that the β-actin and tau mRNAs in granules are transported to axonal growth cones, where they are likely translated (Aronov et al., 2002; Bassell et al., 1998; Zhang et al., 2001).

GAP-43 is a neuronal, developmentally regulated protein (Skene and Willard, 1981c) localized to distal axons and growth cones (Burry et al., 1992; Goslin et al., 1988; Goslin and Banker, 1990; Skene et al., 1986), but not to dendritic growth cones (Goslin et al., 1990; Goslin and Banker, 1990). GAP-43 is proposed to function in axonal growth cones by augmentation of cell adhesion receptor signaling (Meiri et al., 1998; Nakamura et al., 1998; Strittmatter et al., 1995). Expression of GAP-43 has been correlated with axon elongation in developing and regenerating neurons (Skene and Willard, 1981a). When axon growth cones reach their targets, the levels of GAP-43 protein fall as they stop elongation and form presynaptic terminals (Burry et al., 1991; Caroni and Becker, 1992; Meiri et al., 1986; Perrone-Bizzozero et al., 1986). Thus, GAP-43 expression is considered an excellent marker of active axonal growth during development and nerve regeneration.

The expression of GAP-43 is post-transcriptionally regulated by the RNA-binding protein, HuD. HuD is a member of the ELAV/Hu family of RNA-binding proteins and in mammals is expressed only in neurons (Chung et al., 1996; King, 1994; Perrone-Bizzozero and Bolognani, 2002; Szabo et al., 1991). HuD has been shown to bind to GAP-43 mRNA and to stabilize its mRNA (Anderson et al., 2000; Beckel-Mitchener et al., 2002; Chung et al., 1997; Mobarak et al., 2000). HuD has also been implicated in binding mRNAs for other important developmentally-regulated proteins such as tau, c-fos, c-myc, p21(waf1), neuroserpin, MYCN, and acetyl cholinesterase (Aranda-Abreu et al., 1999; Chagnovich et al., 1996; Chung et al., 1996; Cuadrado et al., 2002; Deschenes-Furry et al., 2002; Joseph et al., 1998; Manohar et al., 2002). HuD-containing granules were seen in growth cones of PC12 cells (Aranda-Abreu et al., 1999) and axons of differentiating P19 cells associated with tau mRNA (Aronov et al., 2002). These granules accumulate in growth cones where tau is translated (Aronov et al., 2002). Because HuD stabilizes GAP-43 mRNA and is found in growth cones, it is possible that HuD and GAP-43 mRNA interact in growth cones during neuronal development. Thus, HuD may be involved in stabilizing mRNA in growth cones, which are sites where the GAP-43 protein is found at high levels.

Toward this goal, we investigated whether HuD is localized to growth cones and whether it displays spatial association with GAP-43 mRNA. Following NGF stimulation of PC12 cells, we found that HuD can be localized in growth cones where it accumulates as granules distributed to both the central and peripheral domains. HuD granules also colocalize with GAP-43 mRNA and ribosomes present in these regions. Altogether, these results suggest that HuD may be involved in post-transcriptional regulation of GAP-43 in growth cones.

MATERIALS AND METHODS

Culture

PC12 cells (PC12-N21 clone) were grown as indicated previously (Burry and Perrone-Bizzozero, 1993). For experiments, cultures were rinsed twice in DMEM with 0.5% serum 6 h prior to the beginning of incubations. The chemicals used were: 5 μg/μL cytochalasin D (C-8273; Sigma, St. Louis, MO), 10 μM nocodazole (N-1404; Sigma), 50 μM puromycin (P-7255; Sigma), 5 μM actinomycin D (A1410; Sigma), and 100 ng/mL 2.5S NGF (0100-700; Roche Applied Science, Indianapolis, IN).

Preparation of Monoclonal Antibodies against HuD

To complete this study, an antibody that specifically recognized only HuD was needed. Monoclonal antibodies directed against HuD were generated using the Monoclonal Antibody Facility, Institute of Neuroscience at the University of Oregon. A synthetic peptide corresponding to the N-terminus of HuD (SNNRNCPSPMQTGAC) was synthesized and conjugated to Keyhole Limpet Hemocyanin (KLH). Mice were immunized by intraperitoneal injection of a KLH-HuD peptide conjugate (HuD-KLH; 50 μg/mouse), emulsified in Complete Freund's Adjuvant boosted with conjugate (50 μg/mouse) emulsified in Incomplete Freund's Adjuvant. Sera were then screened for anti-HuD protein antibodies by Western blot analysis using recombinant HuD. The animal with the highest anti-HuD protein titer was then boosted with conjugate (200 μg 4 d before lymphocyte collection, and 100 μg 3 d before collection) in PBS. Lymphocyte collection, hybridoma generation, and subsequent cell culture were performed as previously described (Marusich, 1988). One hybridoma (16C12) was selected on the basis of its reactivity with HuD on immunoblot analysis with recombinant HuD. 16C12 is an IgG2b kappa monoclonal antibody available from Clonegene (Hartford, CT) and was purified from serum-free medium hybridoma culture supernatants.

The specificity of the antibodies was determined in im-

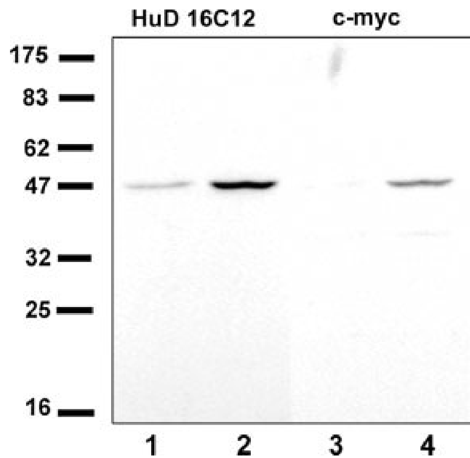

Figure 1 Immunoblots showing the specificity of the 16C12 monoclonal antibody to HuD. Cell lines used: PC12-N21 cells (lanes 1 and 3); PC12-N21 c-myc-HuD (lanes 2 and 4). The 16C12 monoclonal antibody to HuD detected endogenous HuD in PC12-N21 cells (lane 1), and over-expressing wild-type HuD in PC12-N21 c-myc-HuD transfected cells (lane 2). The c-myc antibody detected the HuD construct in PC12-N21 c-myc-HuD transfected cells (lane 4), but did not detect wild-type HuD in PC12-N21 cells (lane 3). Molecular weight markers are indicated on the left in kD.

munoblots with cells expressing wild-type HuD or cells transfected with a construct containing c-myc-tagged HuD (Anderson et al., 2000). In PC12-N21 cells with only wild-type HuD, the anti-HuD 16C12 (Fig. 1, lane 1) recognized a band at 47 kD, and PC12-N21-c-myc-HuD cells over-expressing full-length HuD had higher levels of HuD (Fig. 1, lane 2). In PC12-N21 cells, no band was detected with anti-myc monoclonal (9E10.2; Invitrogen; Fig. 1, lane 3). Also, PC12-N21-c-myc-HuD cells over-expressing full length HuD anti-myc antibodies (Fig. 1, lane 4) recognized a 47 kD band.

Immunoblot

Twenty-five micrograms of total protein were loaded per well in a 10% polyacrylamide gel and transferred to a PVDF membrane. Immunoblotting was done with blocking in Tris-buffered saline (TBS) with 5% milk and 0.05% Tween 20. The membrane was incubated in anti-HuD at 1:1000 for 3 h. Following four rinses the membrane was incubated in goat antimouse IgG-HRP 1:1000 for 1 h. After exposure to ECL Western Blotting Reagent (Amersham Biosciences, Piscataway, NJ), the membrane was exposed to ECL Hyper-film (Amersham Biosciences). Some membranes were stripped in 25 m*M* glycine, 1% SDS, at pH 2.0 for 30 min. Subsequent incubations with sheep anti-GAP-43 (Benowitz et al., 1988) 1:1000, rabbit anti-sheep HRP 1:5000, and anti-ERK 1 (sc-093; Santa Cruz Biotechnology, Santa Cruz, CA) plus anti-ERK 2 (sc-154; Santa Cruz Biotechnology) 1:66 and goat anti-rabbit HRP 1:1000 were done in the same buffers.

Light Microscopic Immunocytochemistry

Cells grown in four-well Permanox Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) were fixed with 2% parafor-maldehyde in phosphate buffer with sucrose for 30 min and rinsed in PBS. The incubation and rinse buffer was 2.5% normal goat serum, 1% BSA, and 0.02% sodium azide (PBS+). Cells were incubated for 2 h in 0.2% Triton X-100 in PBS at room temperature. Incubations with primary an-tibodies were for 3 h and with secondary antibodies for 1 h all at room temperature. The antibodies used were: anti-HuD (16C12; described here) at 1:1000, anti-β-tubulin (C-terminus) 1:2000 (T-5168; Sigma), phalloidin Alexa Fluor 488 20 μL/mL (A-12379; Molecular Probes, Eugene, OR), anti-S6 1:500 (2212; Cell Signaling, Beverly, MA), goat anti-mouse IgG Alexa Fluor 488 1:1000 (A-11029; Molec-ular Probes), goat anti-rabbit IgG Alexa Fluor 488 1:1000 (A-11034; Molecular Probes), goat anti-mouse Alexa Fluor IgG 546 1:1000 (A-11030; Molecular Probes), goat anti-mouse Alexa Fluor IgG 647 1:1000 (A-21245; Molecular Probes). For wide-field microscopy, images were collected on a Zeiss Axioskop with an Olympus MagniFire camera system. Images were also collected on a Zeiss 510 META laser scanning confocal on an Axioplan 2 microscope. The microscopes were located in the Campus Microscopy and Imaging Facility at the Ohio State University.

To quantitate the number of granules in growth cones, confocal images of individual growth cones were evaluated. For each of 30 growth cones, the number of granules was counted that were labeled for GAP-43 mRNA alone, HuD alone, and GAP-43 mRNA and HuD colocalized. The total number of granules for each growth cone was used to calculate the mean and standard errors of the mean (SEM). The number of colocalized granules was tested against either the GAP-43 mRNA granules or the HuD granules with Student's *t* test. For the 3D graph, each growth cone was plotted based on the number of granules labeled for GAP-43 mRNA alone, HuD alone, or GAP-43 mRNA and HuD colocalized.

Fluorescence *In Situ* Hybridization

Four oligos (50 bases each), complementary to sequences of GAP-43 mRNA, were designed using the software program Oligo 4, were checked for lack of homology to other mR-NAs using BLAST search, and were synthesized on a DNA synthesizer (Latham et al., 1994). Each oligonucleotide was modified at five positions within the sequence and chemi-cally labeled using digoxigenin succinamide ester (Roche Molecular Biochemicals) on thymidine. Probes were se-lected from unique regions and were of identical length, guanine/cytosine (GC) content, and hapten incorporation. The probes were antisense to sequences of 158–207, 300–349, 799–848, and 1156–1205 from GAP-43 mRNA (Ac-

cession NM_017195). A scrambled probe and hybridization buffer containing no probe were used as negative controls and showed no labeling. *In situ* hybridization for GAP-43 mRNA was completed as previously described for β-actin mRNA (Bassell et al., 1998).

Cells grown on glass coverslips were fixed in 2% paraformaldehyde and equilibrated in 1X SSC with 40% formamide for 5 min. Each coverslip was incubated at 37°C overnight in hybridization reactions containing 24 ng of oligonucleotide probe, 1X SSC, 40% formamide, 0.4% BSA, 20 m*M* ribonucleotide vanadyl complex, (10 mg/mL), salmon testes DNA, (10 mg/mL), *E. coli* tRNA, and 10 m*M* sodium phosphate. Cells were washed twice with 1X SSC/ 40% formamide for 15 min at 37°C, and then with 1X SSC three times, 10 min each at room temperature. After blocking in TBS+ (50 m*M* Tris, pH 7.4, 150 m*M* NaCl, 0.1% Triton X-100) with BSA (2%) and fetal bovine serum (2%) at 37°C for 1 h, the hybridized probes labeled with digoxigenin were detected using Cy3-mouse monoclonal antibody to digoxigenin (Jackson ImmunoResearch Labs, West Grove, PA) in TBS with 1% BSA at room temperature for 1 h, then washed again in TBS with 1% BSA and incubated in secondary antibody anti-mouse IgG-Cy3 (Jackson ImmunoResearch Labs) for 25 min at room temperature. Cells were then washed successively in TBS-T, $MgCl_2$,and PBS.

Prior to immunocytochemistry labeling for HuD and S6, coverslips were washed in PBS+ for 30 min. To block the anti-mouse antibodies used previously, the coverslips were incubated in 1:20 whole mouse serum (Jackson ImmunoResearch Labs) for 30 min, rinsed, and incubated in donkey anti-mouse Fab (Jackson ImmunoResearch Labs) 20 μg/mL for 30 min, and rinsed in PBS+. Subsequent HuD immunocytochemistry was performed as indicated above. Controls included HuD immunocytochemistry without fluorescent *in situ* hybridization (FISH) and FISH followed by only the secondary Alexa Fluor 543, which were negative. Immunocytochemistry with a nonrelated mouse antibody showed labeling specific to that antibody.

RESULTS

HuD Levels and Distribution in NGF-Stimulated PC12 Cells

Previous studies indicated that the total amount of HuD mRNA and protein does not change within the first 24 h of NGF treatment of PC12 cells (Dobashi et al., 1998; Kasashima et al., 1999; Mobarak et al., 2000; Steller et al., 1996), suggesting that NGF changes the localization rather than the levels of HuD protein in the cell. To show that HuD expression did not change in the first 24 h after stimulation, we used Western blot [Fig. 2(A)]. Beginning at 6 h, HuD protein levels showed a decrease, which returned to control levels at 18 h, and then increased at 24 h. To correlate the levels of HuD with those of GAP-43, the

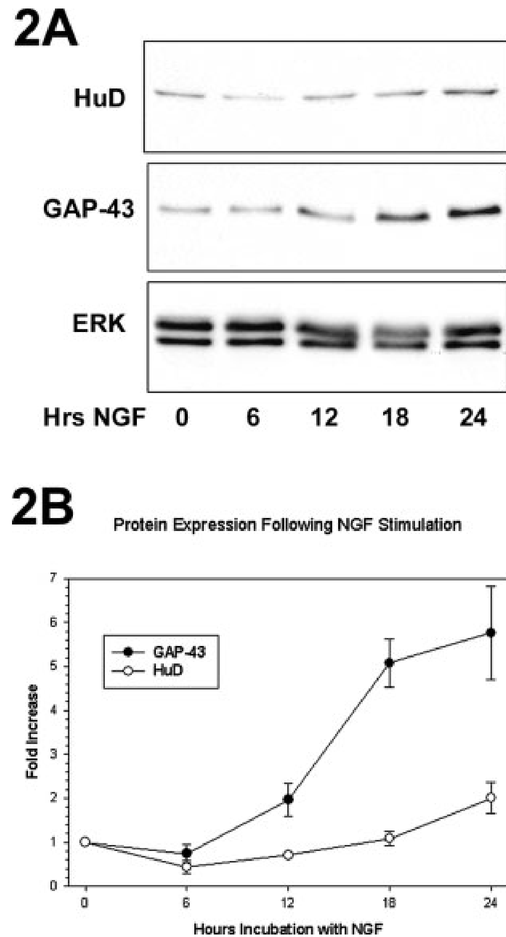

Figure 2 Expression of HuD and GAP-43 proteins in NGF-stimulated PC12-N21 cells. (A) In immunoblots of cultures stimulated with NGF for up to 24 h, HuD shows a decrease in levels at 6 h but later returns to control levels, and at 24 h shows an increase. GAP-43 first increases at 12 h after NGF stimulation. ERK levels were evaluated for loading of the gel. (B) Quantitation of two experiments shows the initial decrease in HuD levels returns to basal levels at 18 h then increases through 24 h. GAP-43 levels remain at basal levels at 6 h, increase at 12 h, and show a strong increase at 18 h. Increases in GAP-43 levels occur after those of HuD.

same blots were reprobed with GAP-43 antibody. In these cells, GAP-43 levels [Fig. 2(A)] first increased at 12 h, followed by increases to 24 h. These results are consistent with the initial growth of neurites stimulated by NGF, which was seen at 18 h (data not shown). This also correlates with the induction of GAP-43 mRNA levels after NGF treatment, which starts after 6 h and peaks at 12 h of NGF treatment (Benowitz and Perrone-Bizzozero, 1991). Finally, the levels of ERK1 and ERK2 were used as a control for loading of the wells [Fig. 2(A)]. Quantitation of results show GAP-43 protein levels increased at early

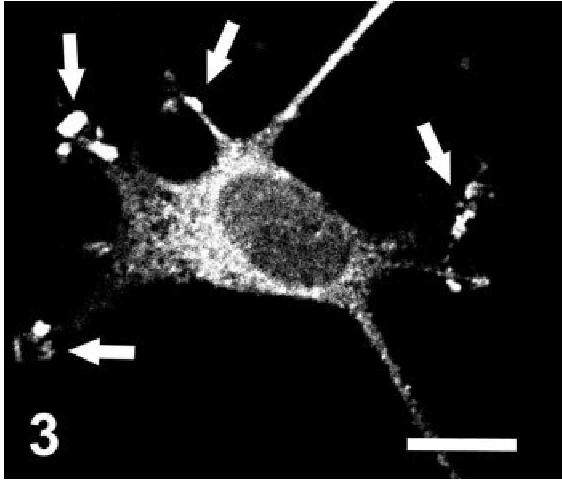

Figure 3 HuD immunocytochemistry in NGF-stimulated PC12 cells. Confocal optical section showing HuD granules in cytoplasm extending into neurites. Growth cones are labeled (arrows) and there is some nuclear labeling. Bar = 10 μm.

time points following NGF stimulation, but the levels of HuD did not increase until after 24 h of treatment [Fig. 2(B)]. These results show that GAP-43 protein levels increase prior to stimulation-dependent increases in HuD.

HuD in NGF-treated PC12 cells appeared as granules (Fig. 3). The finding of HuD in granules is consistent with reports of other ELAV/Hu family members (Antic and Keene, 1998). In the cell body, the HuD granules were localized predominantly at the nuclear envelope in a perinuclear rim. In most cell bodies, HuD-labeled granules accumulated at a higher concentration at one side of the nucleus (Fig. 3). Within the nucleus, HuD labeling was consistently found. NGF-stimulated PC12 cells have many neurites ending in growth cones, which show extensive HuD labeling (Fig. 3, arrows).

HuD in the Central and Peripheral Domains of Growth Cones

Structurally and functionally, the growth cone can be divided into the central domain, an area enriched with microtubules, and the peripheral domain with an extensive network of actin (Forscher and Smith, 1988). To determine if HuD was restricted to the central domain, these cells were analyzed with double labeling immunocytochemistry. Growth cones contained HuD [Fig. 4(A)], which colocalized with tubulin [Fig. 4(B)] in the central domain. A few microtubules did extend into the peripheral domain, but these are proposed to be involved in growth cone turning (Challacombe et al., 1997). HuD granules, however, were seen extending into the central domain. The peripheral domain of growth cones contained large amounts of filamentous actin and labeled extensively with phalloidin [Fig. 4(E)]. In NGF-stimulated PC12 cells, the phalloidin-labeled peripheral domain contained numerous HuD granules [Fig. 4(D)]. The HuD granules not only filled the peripheral domain, but were found in filopodia containing actin filaments and extending from the peripheral domain [Fig. 4(F)]. This result suggests that HuD is associated with actin in the peripheral domain and filopodia.

To show that HuD granules in growth cones are not attached to microtubules, microtubules were disrupted with nocodazole. Treatment with nocodazole for 30 min left the growth cones intact, and microtubules were depolymerized as seen by the loss of linear microtubule labeling in neurites and the diffuse label for tubulin in growth cones [Fig. 4(H)]. In these growth cones, depolymerized tubulin label was now found in the peripheral domain with diffuse labeling for tubulin extending into filopodia. Growth cones in nocodazole-treated cells showed accumulations of HuD positive granules in the peripheral domain [Fig. 4(G)], colocalized with the depolymerized tubulin [Fig. 4(H)], which was seen in the peripheral domain and filopodia [Fig. 4(I)]. These results suggest that in early (30 min) nocodazole-treated cells, the depolymerized microtubules released HuD granules, some of which ended up in growth cone filopodia.

Treatment of cells with cytochalasin D disrupted actin filaments and led to the collapse of the growth cone peripheral domain (Forscher and Smith, 1988). After 30 min treatment with cytochalasin D treatment, filamentous actin labeling was lost and only seen in dense patches [Fig. 4(K)], consistent with previous reports (Challacombe et al., 1996). After cytochalasin D treatment, individual HuD granules were rarely observed, and HuD labeling in the growth cone was mostly found in large patches of granules [Fig. 4(J)]. Thus, HuD granules were withdrawn from filopodia with depolymerized actin and accumulated in the growth cone, where individual granules could not be seen [Fig. 4(J)]. These results indicate that HuD binds directly or indirectly to filamentous actin and that in the growth cone, where actin is the major cytoskeletal element, HuD granules were bound to actin and that microtubules were not bound to HuD granules.

The presence of HuD granules in the peripheral domain suggested that mRNA bound to HuD could be translated at these sites. Ribosomes are a component of granules containing the RNA binding proteins Staufen (Agalarov et al., 2000; Krichevsky and Kosik, 2001) and HuB (Antic and Keene, 1998). To deter-

mine if ribosomes were present, the distribution of a ribosomal protein was observed with an antibody that recognizes the S6 protein component of the 40S ribosomal subunit. In NGF-stimulated cultures, HuD was localized to both the central and peripheral domains [Fig. 4(M)]. However, the S6 protein was predominantly located in the central domain, with the central domain and filopodia showing little S6 labeling [Fig.

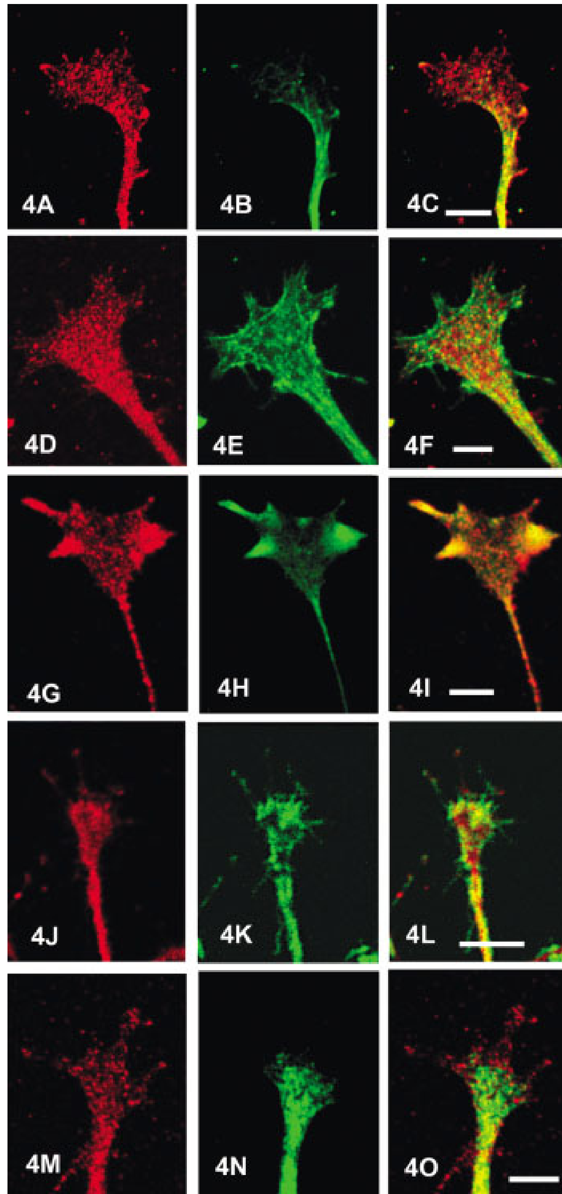

Figure 4

Figure 4 Distribution of HuD in growth cones. All cells were stimulated for 24 h with NGF, some were treated with nocodazole (G,H,I), or were treated with cytochalasin D (J,K,L). HuD labeling (A,D,G,J,M). Tubulin labeling (B,H). Actin labeling (E,K). Ribosomal labeling S6 (N). Merged images (C,F,I,L,O). (A,B,C) HuD granules are found through the entire growth cone (A), but microtubules (B) are restricted to the neurite and central domain. (D,E,F) Both HuD granules (D) and actin filaments (E) are distributed in the peripheral domain of growth cones. (G,H,I) Following treatment with nocodazole to depolymerize microtubules, HuD granules (G) remain in the peripheral and central domains while microtubules are depolymerized and tubulin (H) is seen diffusely throughout the growth cone. (J,K,L) Cytochalasin D depolymerizes actin filaments causing loss of the actin network in the peripheral domain (K) and change in HuD (J) distribution from granules to a diffuse distribution. (M,N,O) Ribosomal protein S6 is present in the central domain, and not in the peripheral domain or filopodia (N). Bars = 5 μm.

Figure 5 FISH for GAP-43 mRNA. (A) GAP-43 FISH and phase optics show GAP-43 mRNA in both the central and peripheral domains of growth cones. However, no GAP-43 mRNA was detected in filopodia [(A), arrows]. Wide-field fluorescence and phase. Bar = 5 μm. (B,C,D) FISH for GAP-43 mRNA combined with HuD immunocytochemistry and confocal microscopy. (B) HuD granules in growth cones were seen in the central domain, peripheral domain, and in filopodia. (C) GAP-43 mRNA was found in the central and peripheral domains. (D) Merged image with yellow indicating double-labeled granules. The arrow indicates a filopodium with HuD label but little GAP-43 mRNA. Bar = 5 μm.

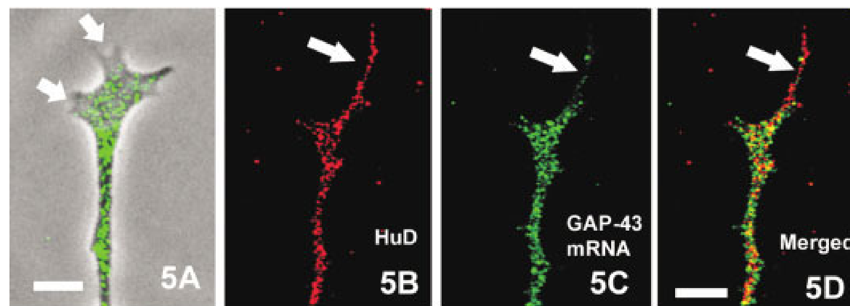

Figure 5

4(N)]. In examining the coincidence of HuD and S6 labeling, most of the S6 labeling was colocalized with HuD in the central domain, with little colocalization in the peripheral domain [Fig. 4(O)]. These results suggested that in the growth cone central domain HuD was colocalized with ribosomes, but in the peripheral domain and filopodia HuD was not found associated with ribosomes.

To evaluate granules associated with both mRNA and ribosomes, puromycin was used to dissociate the translation complex. In PC12 cells treated for 3 h with puromycin, elongation of neurites was compromised, but intact HuD granules were found in growth cones (data not shown). In many of the growth cones, the peripheral domains had collapsed and much of the actin filament network was disorganized even though some filopodia and HuD granules were still seen. In addition, inhibition of transcription with actinomycin D at 6 h showed no change in size or distribution of the HuD granules (data not shown). These results suggest that dissociation of ribosomes from mRNA with puromycin had little effect on HuD granules.

GAP-43 mRNA and HuD in Growth Cones

GAP-43 mRNA has been shown to be stabilized by HuD binding in PC12 cells (Mobarak et al., 2000). For HuD to function in growth cones, it is necessary to show that both HuD and GAP-43 mRNA are present in growth cones. To evaluate the distribution of GAP-43 mRNA bound to HuD granules, FISH was used. Growth cones of PC12 cells showed GAP-43 mRNA distributed as granules similar in size and shape to the HuD granules [Fig. 5(A)]. This is the first report showing GAP-43 mRNA localized to growth cones. These granules were found in both the central and peripheral domains, however, few granules were seen in filopodia [Fig. 5(A), short arrows]. The overall distribution of GAP-43 mRNA was very similar to that of HuD granules, and FISH for GAP-43 mRNA in combination with immunocytochemistry for HuD was performed to evaluate colocalization. In growth cones, granules labeled for HuD were found both separate and colocalized with granules labeled for GAP-43 mRNA [Fig. 5(B–D), yellow]. Within the central and peripheral domains, many of the HuD granules were colocalized with GAP-43 mRNA granules, while some of the GAP-43 mRNA granules were not colocalized. Filopodia, which labeled with HuD, had little GAP-43 mRNA [Fig. 5(B–D), arrow].

To evaluate the granules in individual growth cones, counts were performed for the number of granules labeled for HuD, GAP-43 mRNA, or both. Re-

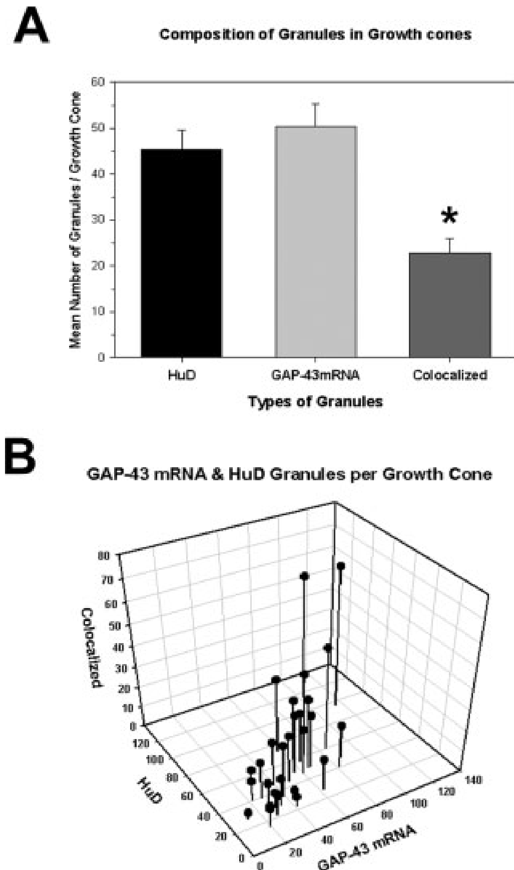

Figure 6 Distribution of HuD and GAP-43 mRNA granules in growth cones. (A) Mean number of HuD granules (HuD), GAP-43 mRNA granules (GAP-43 mRNA), and colocalized granules (colocalized) with SEM indicated. The numbers of HuD and GAP-43 mRNA granules were similar with no statistical difference. The numbers of colocalized granules were half of the numbers of HuD or GAP-43 mRNA granules (* $p < 0.001$). (B) A 3D plot shows labeling of individual growth cones. The xy plot for HuD and GAP-43 mRNA shows similar numbers of granules in growth cones (x axis HuD and y axis GAP-43 mRNA). The lack of growth cones close to either axis confirms this observation. The numbers of colocalized granules (z axis, colocalization) increased in proportion to the number of both types of granule.

sults showed that the number of granules containing both GAP-43 mRNA and HuD [Fig. 6(A), Colocalized] was approximately half of the number of granules in either population. However, it was not clear whether these results were due to growth cones containing unequal amounts of HuD and GAP-43 mRNA. To evaluate this further, we used a 3D plot [Fig. 6(B)]. Individual growth cones had similar numbers of granules labeled for either HuD or GAP-43 mRNA as seen by the clustering in the center of the graph, and the lack of points near either the HuD or the

GAP-43 mRNA axes [Fig. 6(B)]. Granules with both HuD and GAP-43 mRNA [Fig. 6(B), Colocalized axis] increased in proportion to the number of granules with individual labels. Thus, in individual growth cones approximately equal numbers of granules containing either HuD or GAP-43 mRNA were found and about half of these granules were colocalized.

In PC12 cell preparations stabilization of GAP-43 mRNA was found in the presence of HuD and polysomes (Beckel-Mitchener et al., 2002), suggesting that HuD may interact with polysomes bound to GAP-43 mRNA. To evaluate the association of HuD and GAP-43 mRNA with ribosomes, ribosomes were labeled with an antibody to the S6 protein, and were compared for the distribution of this protein to that of HuD and GAP-43 mRNA [Fig. 7(A–C) show single label]. GAP-43 mRNA granules were found in both the central and peripheral domains, but not in filopodia [Fig. 7(A)]. Three granules are indicated at the white arrows [Fig. 7(A)] and were about half a micrometer in diameter. HuD granules were seen throughout the growth cone [Fig. 7(B)]. S6 granular labeling for ribosomes was seen at highest concentration in the growth cone central domain [Fig. 7(C)] and was not as extensive as that for either GAP-43 mRNA or HuD.

To investigate the colocalization of granules for GAP-43 mRNA, HuD, and S6, these images were merged [Fig. 7(D–G)]. As seen previously, many of the HuD granules were also labeled for GAP-43 mRNA in the merged yellow granules [Fig. 7(D), arrows right and left side of growth cones]. To evaluate the ribosomes' distribution, the S6 and GAP-43 mRNA images were merged [Fig. 7(E)] with only a small amount of blue S6 label not colocalized with the green GAP-43 mRNA label. The arrows on the right side of the growth cone indicate GAP-43 mRNA granules that colocalize with S6 for ribosomes and have a light green color, indicating colocalization. To evaluate the distribution of S6 and HuD images were merged [Fig. 7(F)].

The merged image revealed that most of the S6 protein was colocalized with HuD as seen by the pink granules [Fig. 7(F), right arrows], but the HuD and GAP-43 mRNA distribution was more extensive, as seen by granules that did not colocalize with S6 [Fig. 7(F), left arrow]. Colocalization of ribosomes with HuD and GAP-43 mRNA was seen when all three labels were merged [Fig. 7(G)]. The white color [Fig. 7(G), right arrows] indicated the localization of all three markers in the central domain of the growth cone, demonstrating an extensive association of HuD and GAP-43 mRNA with ribosomes in this region. Most ribosomal S6 labeling was colocalized with either HuD or GAP-43 mRNA as seen by the lack of blue labeling. However, some of the GAP-43 mRNA granules, seen as green labeling, did not colocalize with either HuD or ribosomes. In addition, HuD granules (red labeling, mainly in the filopodia) did not colocalize with either GAP-43 mRNA or ribosomes. Previous studies have shown that HuD bound to and stabilized GAP-43 mRNA in PC12 cells (Anderson et al., 2000; Chung et al., 1997; Mobarak et al., 2000) and in a polysome-based cell free mRNA decay assay (Beckel-Mitchener et al., 2002). Our results showed that most of the ribosomes in growth cones were associated with HuD and GAP-43 mRNA, consistent with the role of HuD binding and stabilizing GAP-43 mRNA in the presence of ribosomes.

DISCUSSION

HuD and GAP-43 mRNA Role in Growth Cones

Our results show for the first time that GAP-43 mRNA is present in growth cones. This observation was extended by the association of GAP-43 mRNA, its binding protein HuD, and ribosomes in growth cones. A role for HuD in stabilizing GAP-43 mRNA has been shown (Chung et al., 1997; Kohn et al., 1996; Tsai et al., 1997), and our observations allow the suggestion that this stabilization can occur in growth cones. Thus, a major conclusion of our study is that the findings are consistent with the hypothesis that translation of mRNA in growth cones is a mechanism whereby levels of GAP-43 protein in neuronal growth cones can be controlled locally.

The expression of GAP-43 is important for neuronal differentiation. Selective distribution of GAP-43 in elongating axons is the result of developing neuronal polarity. During neuronal differentiation, GAP-43 protein is lost from the cell body, distributed to distal axons, and concentrated in growth cones (Burry et al., 1991, 1992; Goslin et al., 1988, 1990; Przyborski and Cambray-Deakin, 1994). Transport of GAP-43 protein associated with vesicles from the cell body to growth cones has been well documented (Benowitz et al., 1981; Skene and Willard, 1981a,b,c). Evidence presented here suggests that in addition to GAP-43 protein being transported to growth cones, the GAP-43 mRNA is also targeted to growth cones, where it could be translated locally. Cell bodies and long axons of developing neurons loose GAP-43 protein, suggesting that growth cone GAP-43 is synthesized by translation, without depending on rapid axonal transport from the cell body. Local control of

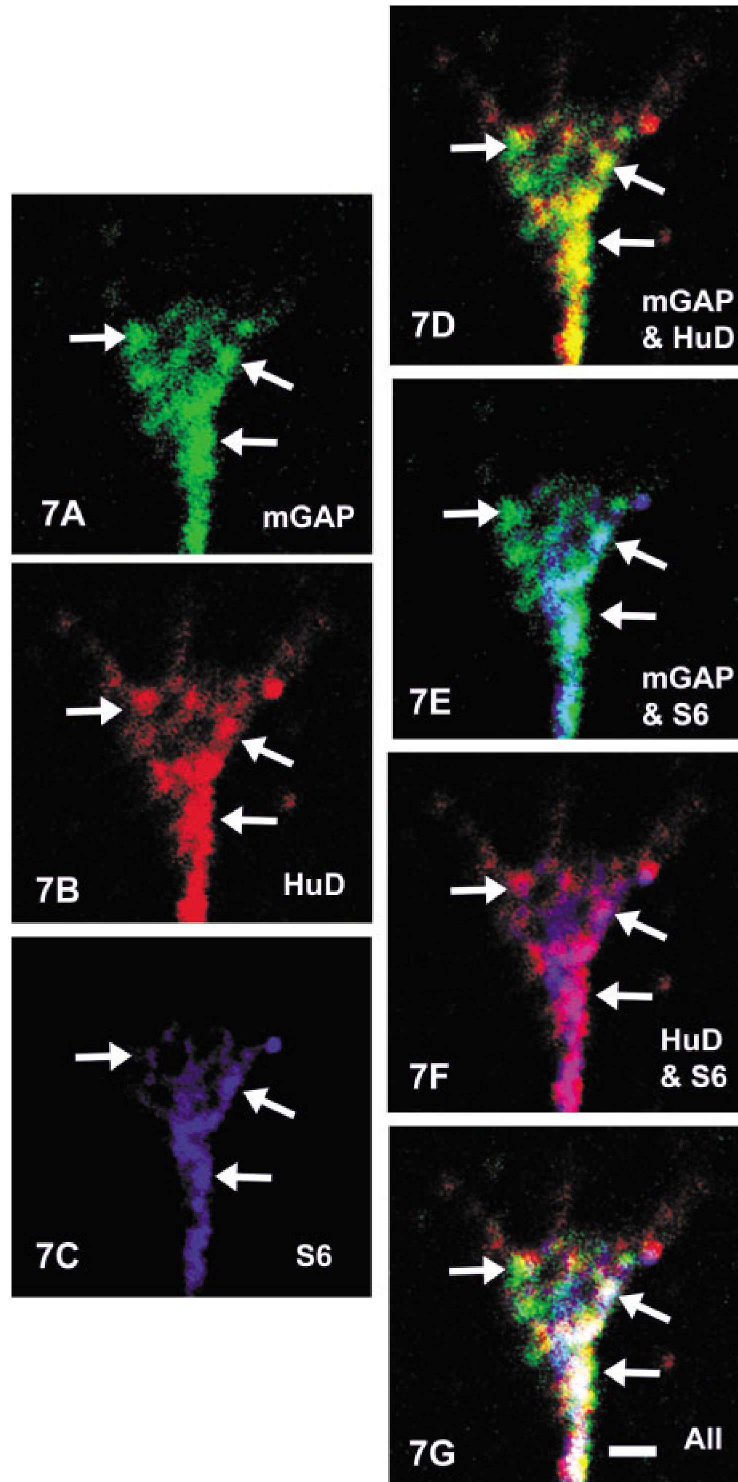

Figure 7 Ribosomes associated with HuD and GAP-43 mRNA. Single label confocal images (A,B,C) of a growth cone showing labeling for GAP-43 mRNA [(A), mGAP], HuD (B), and S6 for ribosomes (C). Arrow on the left of the growth cone indicates a granule colocalized for GAP-43 mRNA and HuD. Arrows on the right of the growth cone indicate granules that colocalize GAP-43 mRNA, HuD, and ribosomes. Merged images of the same growth cone (D,E,F,G). (D) Merged image showing GAP-43 mRNA (mGAP, green) and HuD (HuD, red). (E) Merged images showing GAP-43 mRNA (mGAP, green) and ribosomes (S6, blue). (F) Merged images showing HuD (HuD, red) and ribosomes (S6, blue) colocalized (light blue). (G) Merged images showing HuD (red), GAP-43 mRNA (green), and ribosomes (S6, blue), and all three colocalized (white). Bar = 1 μm.

translation in growth cones has been shown to be a powerful mechanism for controlling axonal growth and guidance (Campbell and Holt, 2001), and thus it is enticing to propose that RNA binding proteins might be involved in this process.

Zipcode binding protein 1 (ZBP1) is an example of a RNA binding protein that is known to bind the zipcode element in the 3′UTR of β-actin mRNA and target it to the growth cone for translation (Bassell et al., 1998). Interestingly, the zipcode element is involved in targeting, but not stabilizing, the β-actin mRNA (Kislauskis et al., 1994). ZBP1 in neurons was found to colocalize in granules within axons, growth cones, and dendrites (Tiruchinapalli et al., 2003; Zhang et al., 2001). Antisense to the zipcode disrupted the formation of a ZBP1 complex with the β-actin mRNA zipcode and prevented the localization of β-actin mRNA in response to NT-3 (Zhang et al., 2001). Such neurons exhibited impaired movements of growth cones and often displayed retractive behavior (Zhang et al., 2001). The ZBP1 was involved in targeting of β-actin mRNA to neuronal growth cones and ultimately to control the amount of cytoskeletal elements necessary for elongation.

The finding that the RNA binding protein HuD is associated with GAP-43 mRNA in growth cones suggests that stabilization and/or targeting of GAP-43 mRNA occurs in growth cones. With the proposed function of GAP-43 as modulating signaling within the growth cone (Nakamura et al., 1998; Strittmatter et al., 1994), rapid modulation of GAP-43 protein levels could be important. One method of altering GAP-43 signaling is to modify the levels of protein by regulating the translation of GAP-43 mRNA. This role is consistent with protein synthesis-dependent turning behavior of the Netrin-1 receptor signaling (Campbell and Holt, 2001; Ming et al., 2002) and the commissural axon synthesis of EphA2 receptors after crossing the midline of the spinal cord (Brittis et al., 2002). However, the demonstration of RNA binding proteins playing a role in neuronal growth cone signaling has not been shown, so the association of HuD with GAP-43 mRNA in the neuronal growth cone suggests that specific RNA binding proteins could play a role in regulation of other mRNAs, such as those for the Netrin-1 receptor.

HuD and GAP-43 mRNA in the Growth Cone Peripheral Domain

Growth cones have two domains, a central domain containing microtubules, mitochondria, and vesicles, and a peripheral domain enriched in filamentous actin with filopodia extending from the leading edge (Bridgman and Dailey, 1989; Forscher and Smith, 1988; Letourneau and Ressler, 1984). The actin network in the peripheral domain functions in axon elongation to direct the growth cone by regulating the assembly and disassembly of actin filaments (Bamburg, 2003). Filopodia, extensions of the peripheral domain, are linked to axon recognition and turning as a result of differences in the substrate (Challacombe et al., 1996).

Axonal growth cones are not thought to contain mRNA, but there are several studies that show it is distributed in growing axons. mRNA has been detected in growth cones of cultured cortical neurons with the dye SYTO 14, which labels mRNA (Knowles et al., 1996). Also, ribosomes, translation initiation factors, and rRNA were found in dorsal root ganglion neuron growth cones (Zheng et al., 2001). Active translation of mRNA was found to be required for growth cone responses in pathfinding (Campbell and Holt, 2001), and for adaptive responses to netrin (Ming et al., 2002). These reports indicate that in some cases growth cones contain mRNA.

While not explicitly examined in previous studies, granules containing mRNA and/or RNA-binding proteins can be seen in different domains within the axonal growth cone. ZBP1 colocalized with β-actin mRNA in growth cones and apparently was restricted to the central domain (Zhang et al., 2001). Tau mRNA localized to growth cones was distributed into what appeared to be the central domain, the peripheral domain, and filopodia (Aronov et al., 2001). CPEB distributed into the central domain and apparently into the peripheral domain of embryonic chick spinal neurons *in vivo* (Brittis et al., 2002). Thus, the distribution of selected mRNA and RNA-binding proteins to the central domain of growth cones was seen in micrographs from previous articles, but this distribution was not specifically mentioned by the authors in these articles.

The previous studies of mRNA in growth cones did not address the distribution of mRNA within the various domains of the growth cone. Results presented here show that ribosomes and microtubules are concentrated in the central domain of the growth cone, but HuD granules and GAP-43 mRNA were distributed in the central domain and the peripheral domain. In addition, HuD was seen in filopodia. Given the lack of peripheral domain organelles and the importance of filopodia in determining growth cone directional movement, it is difficult to understand why the RNA-binding protein HuD and GAP-43 mRNA were present in the actin-rich peripheral domain. A role for actin filaments in HuD localization in the peripheral domain suggested by our

results showed coalescence of HuD granules following depolymerization of actin filaments. Our results were consistent with reorganization of HuB granules in growth cones treated with cytochalasin (Antic and Keene, 1998). Results in Figure 4 showed that disruption of microtubules with nocodazole did not affect the size or the overall distribution of HuD granules in PC12 cell growth cones. These results are similar to those reported for HuD (Behar et al., 1995) or for HuB (Antic and Keene, 1998). Taken together, these results and the previous studies indicate a role for actin in the localization of granules in growth cones, rather than a role for microtubules in localization of granules in axons (Olink-Coux and Hollenbeck, 1996). In addition to reports that showed that microtubules transport HuD granules in neurites, our results show actin might be responsible for HuD distribution in growth cone peripheral domains.

HuD Granules and mRNA Granules

The colocalization of HuD and GAP-43 mRNA in growth cone granules supports the concept that RNA-binding proteins are able to localize selected mRNA to growth cones. In growth cones, our results showed that half of the total HuD granules and half of the total GAP-43 mRNA granules were associated. In other systems, similar results were seen with RNA-binding proteins and mRNA. Results with ZBP1 showed that while significant colocalization of ZBP1 and β-actin mRNA occurred, spatially distinct granules were also seen to contain either ZBP1 or β-actin mRNA (Zhang et al., 2001). HuD and tau mRNA granules in P19 cells were highly colocalized (Aronov et al., 2002), but only a portion of HuB colocalized with ribosomes in cultured cortical neurons (Gao and Keene, 1996). This lack of complete association of the RNA-binding protein and mRNA suggests that the association is more complex involving other components.

Granules containing mRNA binding proteins and mRNAs are also associated with polysomes. Previous reports with HuB and HuR have shown association with ribosomes (Gallouzi et al., 2000; Rudnicki et al., 1989). Colocalization of HuD and ribosomal RNA in dorsal root ganglion neuron cell bodies has been reported (Anderson et al., 2003). Increases in GAP-43 mRNA half-life were found in a cell-free polysome-based decay system when HuD was present (Beckel-Mitchener et al., 2002). Thus, the report here that the high level of association found for ribosomes and HuD in growth cones is strong support for the presence of ribosomes in granules that can stabilize GAP-43 mRNA.

ELAV/Hu family members were found as granules containing other mRNA-binding proteins and mRNA (Antic and Keene, 1998). The distribution of mRNA in axons and growth cones was in the form of granules that were colocalized with ribosomal proteins and cytoskeletal proteins (Knowles et al., 1996). Density gradients demonstrated that the mRNA binding protein HuB was found in developing cultured neurons in two types of complexes: those complexes bound to mRNA and microtubules, and those complexes with ribosomes bound to both mRNA and microtubules. Puromycin shifts this balance away from complexes with ribosomes and toward complexes without ribosomes that retained their granular appearance (Antic and Keene, 1998). Puromycin release of ribosomes from mRNA has been seen in neurons with movement of mRNA from cell bodies to dendrites (Kleiman et al., 1993; Lu et al., 1998). The results here showed that in puromycin-treated growth cones, HuD granules were still present, even with the collapse of the growth cone peripheral domain, in contrast to disruption of the actin network, which forms large patches of HuD. Thus, binding of mRNA with ribosomes appears not to be required for maintenance of growth cone HuD granules, even though ribosomes were closely associated with HuD granules in growth cones. The relationship of GAP-43 mRNA, HuD, and ribosomes is complex and additional studies are needed to understand it.

In conclusion, our results indicate that in the growth cone, granules containing the RNA-binding protein HuD and GAP-43 mRNA were also associated with ribosomes. Furthermore, we have recently shown that in DRG neurons, HuD is also localized to granules in association with both the GAP-43 mRNA and with ribosomal RNA (Anderson et al., 2003). Given that polysomes participate in the control of mRNA stability (Beckel-Mitchener et al., 2002), our finding of HuD colocalization with GAP-43 mRNA in ribosomes supports the idea that HuD is involved in the local control of mRNA stability in growth cones.

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