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Nerve terminals of squid photoreceptor neurons contain a heterogeneous population of mRNAs and translate a transfected reporter mRNA

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Abstract

It is now well established that the distal structural/functional domains of the neuron contain 2a diverse population of mRNAs that program the local synthesis of protein. However, there is still a paucity of information on the composition and function of these mRNA populations in the adult nervous system. To generate empirically, hypotheses regarding the function of the local protein synthetic system, we have compared the mRNAs present in the squid giant axon and its parental cell bodies using differential mRNA display as an unbiased screen. The results of this screen facilitated the identification of 31 mRNAs that encoded cytoskeletal proteins, translation factors, ribosomal proteins, molecular motors, metabolic enzymes, nuclear-encoded mitochondrial mRNAs, and a molecular chaperone. Results of cell fractionation and RT-PCR analyses established that several of these mRNAs were present in polysomes present in the presynaptic nerve terminal of photoreceptor neurons, indicating that these mRNAs were being actively translated. Findings derived from *in vitro* transfection studies established that these isolated nerve terminals had the ability to translate a heterologous reporter mRNA. Based upon these data, it is hypothesized that the local protein synthetic system plays an important role in the maintenance/remodelling of the cytoarchitecture of the axon and nerve terminal, maintenance of the axon transport and mRNA translation systems, as well as contributing to the viability and function of the local mitochondria.

Introduction

Although the majority of neuronal mRNAs are transcribed and translated in the neuronal cell soma, a subset of these gene transcripts are selectively transported to the distal structural/functional domains of the neuron to include the dendrite, axon, and presynaptic nerve terminal (reviewed in Alvarez *et al*., 2000; Giuditta *et al*., 2002). Early findings derived from the squid giant axon, used as a model invertebrate motor neuron, indicated that the axon contained as many as 100–200 different polyadenylated mRNAs, a value that represented $< 1\%$ of the total mRNAs expressed in the parental cell bodies (Perrone Capano *et al*., 1987). Results of quantitative RT-PCR analyses established that the relative abundance of these mRNAs in the axon differed markedly from that in the cell soma, a finding that suggested that these gene transcripts were being differentially transported into the axonal compartment (Chun *et al*., 1996). Consistent with these early findings, a recent, unbiased screen of a cDNA library prepared from isolated *Aplysia* sensory neuron processes revealed the presence of over 260 distinct mRNAs (Moccia *et al*., 2003). Similar to the situation in the squid giant axon (Kaplan *et al*., 1992), the library was enriched for mRNAs encoding cytoskeletal elements and components of the translational machinery. The subcellular compartmentation of these mRNAs is thought to program the local synthesis of proteins that play a key role in the function of the axon and nerve terminal to include, navigation of the growth cone (Campbell & Holt, 2001; Ming *et al*., 2002; Zhang & Poo, 2002), synapse formation (Schacher & Wu, 2002), axon regeneration (Zheng *et al*., 2001; Hanz *et al*., 2003), and the synthesis of membrane receptors employed as axon guidance molecules (Brittis *et al*., 2002).

In invertebrate model systems, there is also evidence to indicate that the local synthesis of protein in the presynaptic nerve terminal plays a key role in activity-dependent synaptic plasticity, such as long-term facilitation (Martin *et al*., 1997; Casadio *et al*., 1999; Beaumont *et al*., 2001; Liu *et al*., 2003). In this regard, the axon might share much in common with dendrites, where local protein synthesis is involved in growth and synaptic plasticity (reviewed in Steward & Schuman, 2003).

To augment our understanding of the possible function(s) that the local protein synthetic system might play in the adult nervous system, we have employed differential mRNA display methodology to characterize more fully the components of the axonal mRNA population. By identifying individual axonal mRNAs, we hoped to generate empirically, hypotheses related to the function of this unique mRNA population. In this communication, we provide the initial results of this unbiased screen and extend our findings to the presynaptic nerve terminals of a sensory neuron. Based upon these findings, it is postulated that local protein synthesis underlies the

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maintenance of axonal and nerve terminal architecture, maintains the axon transport and mRNA translation systems, and contributes to the viability and function of the local mitochondrial population.

Materials and methods

Tissue preparation and RNA isolation

Squid (*Loligo pealii*) were obtained during the summer at the Marine Biological Laboratory (Woods Hole, MA, USA). The giant fibre lobe (GFL), containing the cell bodies of the giant axon, was hand-dissected from the stellate ganglion and the axoplasm extruded from the isolated giant axon using a Teflon roller (Giuditta *et al*., 1991). A synaptosomal fraction was obtained from the squid optic lobe as described by Crispino *et al*. (1997). Polysomes were isolated from the optic lobe and synaptosomes by centrifugation of a detergent-treated postmitochondrial supernatant through 2.0 M sucrose and purified by linear sucrose density gradient centrifugation as previously described (Crispino *et al*., 1997; Gioio *et al*., 2001). RNA was isolated from all tissue and cell fractions using TRIZOL reagent (Invitrogen).

Differential mRNA display and dot-blot hybridization analysis

Experiments were performed using the Delta Differential Display Kit (Clontech) following recommended protocols. First-strand cDNA synthesis was accomplished using total RNA (1 μg) combined with one of the nine anchored oligo(dT) primers provided and Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase. [^{33}P]-labelled amplicons were generated from the cDNA by PCR using pair-wise combinations of the nine anchored oligo (dT) primers and 10 arbitrary primers, Advantage Klentaq Polymerase, and TaqStart Antibody (Clontech). Three low-stringency cycles were conducted at 40 °C followed by 25 high-stringency cycles at 60 °C. PCR products were displayed by electrophoresis on 5% polyacrylamide-urea gels and were visualized by exposure to Biomax MR X-ray film (Kodak). cDNAs preferentially amplified from axoplasmic RNA were excised from the gels, and were reamplified using the same primer sets. Reamplified cDNA was subsequently ligated into the vector pGEM Eazy (Promega) and cloned using standard procedures. The fidelity of the clones was established by amplification of the insert using the original primer sets and comparing the size of the PCR product to the size of the amplicons excised from the gel.

For dot-blot hybridization analysis, cDNA representing each clone (10 ng) was blotted onto Nytran Plus membrane (Schleicher & Schuell) and was hybridized to [^{32}P-labelled rat brain cDNA prepared from total poly(A)+RNA by random oligonucleotide priming using Superscript RT (Gibco BRL, Rockville, MD, USA). Reactions were conducted in 0.6 M NaCl, 1% SDS, 100 μg/mL *E. coli* sheared DNA at 55 °C for 16 h. The filter blot was then washed to a final stringency of 0.5× SSC at 55 °C and was exposed to X-ray film for 16 h.

DNA sequencing

Sequencing of the cDNA clones was carried out on a PE Applied Biosystems model 310 sequencer (Foster City, CA, USA) using Big Dye Terminator Cycle Sequencing Ready Reaction. To extend the sequence of the primary cDNA clones, we employed both 5′- and 3′-RACE technology using the First Choice RLM-RACE kit (Ambion). For 5′-RACE, squid total RNA was treated with calf intestinal phosphatase and the 5′-methyl CAP structure removed from full-length mRNA using Tobacco acid pyrophosphatase. A 45 oligonucleotide adapter was subsequently affixed to the 5′-end of the mRNAs using T4 RNA ligase. To amplify the 5′-ends, the mRNAs were PCR amplified using gene specific primers in combination with a primer complimentary to the 5′-Race Adapter. cDNAs extended by 3′-RACE were reverse transcribed from optic lobe total RNA using an oligo(dT)$_{12}$ attached to the 3′-RACE Adapter. The transcripts were PCR amplified using a primer specific for the 3′-RACE Adapter and a gene-specific primers. The RACE-extended cDNAs were subsequently inserted into pGEM-T vector (Promega) for sequencing. Comparative sequence analyses were conducted using the Blast X program to screen GENEBANK and SWISSPRO databases.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Reverse transcription was conducted using oligo(dT) primers, the Superscript Preamplification System (Gibco BRL), and 1.0 μg of optic lobe cytoplasmic or synaptosomal total RNA. In experiments that employed fractionated polysomal RNA, 25% of the sample recovered from both the monosomal and polysomal portions of the linear sucrose gradients were used in the analysis. Amplification was accomplished using gene-specific primers for the following squid mRNAs:
β-actin
(forward) 5′-CCTGCTATGTATGTGGCTAT,
(reverse) 5′-AAGCACTTCCTATGGACGAT;
β-tubulin
(forward) 5′-TGGTGCTAAGTTCTGGGAAG,
(reverse) 5′-CCAATAAAAGTAGCCGACAT;
neurofilament protein,
(forward) 5′-CTTATGATTTTAGCTATGGAG,
(reverse) 5′-TCCTTAATAGCAGAGGTTTCT;
and elongation factor 2 (EF-2),
(forward) 5′-CCGATCTTTTTGGTTATGCCG,
(reverse) 5′-CGAAGTCTTAGGTTTTATTACGTGCG.
In all reactions, 35 rounds of amplification were conducted at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s.

Reporter mRNA transfection analysis

A transcription system for the generation of green fluorescent protein (GFP) mRNA was constructed by restriction digest of the pEYFP-Mito vector (Clontech), which contained a GFP cDNA insert. The DNA fragment was ligated into the BlueScript SK-phagemid (Stratagene) containing promoter sequences for T7 and T3 RNA polymerase. Sense and antisense mRNAs were transcribed and the biological activity of the transcripts was initially assessed by translation in a rabbit reticulocyte cell-free translation system (Promega). GFP translation products were identified by Western blot analysis using a monoclonal GFP antibody conjugated to horse radish peroxidase (HRP) (JL-8, 1 : 200 dilution; BD Biosciences Clonetech). The GFP mRNA was transfected into squid optic lobe synaptosomes, which were resuspended in artificial seawater, using a lipid-based transfection system (NeuroPorter Transfection Reagent, Gene Therapy Systems). The synaptosomes where then diluted with artificial seawater and spotted onto poly-lysine coated coverslips. The translation of the GFP mRNA was monitored either by autofluorescence or by immunohistochemistry using a monoclonal GFP antibody and a rhodamine-labelled donkey antimouse secondary antibody (1 : 100 dilution; Chemicon). Affinity purified antibodies to a-type synapsin were the kind gift of Dr A.J. Czernick (Rockefeller University, NY,

USA) and reaction products visualized using a FITC-labelled secondary antibody.

Results

Identification of axonal mRNAs

Previously, we reported that the squid giant axon contained a diverse population of mRNAs that encoded key elements of the cytoskeleton, axon transport systems and local energy metabolism (Kaplan *et al*., 1992; Gioio *et al*., 1994; Chun *et al*., 1995). In each case, the axonal localization of these mRNAs was confirmed by *in situ* hybridization histochemistry. To further define the composition of this mRNA population, we used differential mRNA display to compare mRNAs present in the giant axon and its cell bodies located in the GFL. The design employed in this experiment is illustrated in Fig. 1. Display experiments were conducted on replicate RNA preparations from both GFL and extruded axoplasm (10 tissue samples/preparation). PCR amplicons that revealed a higher relative abundance in the axon ($n = 150$) were excised from the polyacrylamide sequencing gels, PCR amplified using the same primer sets, and affixed to nylon filter supports. The cDNAs were subsequently screened for evolutionary sequence conservation, at the nucleotide level, by dot-blot hybridization using radiolabelled rat brain cDNA as a probe. Interestingly, approximately 25–30% of the squid axoplasmic cDNAs cross-reacted to the rat brain probe under conditions of moderate hybridization stringency (Fig. 1). Hence, in principle, these mRNAs could encode proteins that serve a fundamental function in both the invertebrate and mammalian nervous systems. Fifty of the cross-reacting cDNAs were selected for sequence analysis to establish their identities. Results of the comparative sequence analysis of these cDNA clones are provided in Table 1. The high level of sequence conservation for these cDNAs was manifest by BLAST X results in terms of *E*-values that ranged from 2.1 to 0. [The *E*-value is the number of hits expected by chance when searching a database.] The percentage sequence similarity between the squid mRNA sequences and the closest related species ranged from 59 to 100% at the amino acid level. An example of this extensive evolutionary sequence conservation is illustrated in Fig. 2. In this case, EF-2 shared 58% sequence identity to its human orthologue with some domains (e.g. amino acid residues 98–155) manifesting 100% identity. Considering conservative amino acid substitutions, the overall sequence similarity between these two proteins was 67%. A maximum value of 79% sequence similarity for squid EF-2 was obtained with the tiger centipede protein (Table 1; *E*-value, $2 \times e^{-99}$).

Among the 50 mRNA transcripts that were preferentially expressed in the axon the identities of 36 of the cDNA clones were established after the initial sequence analysis. Of these clones, 27 represented independent nuclear-encoded mRNAs, whereas four mRNAs were encoded by the mitochondrial genome: NADH dehydrogenase, cytochrome *c* oxidase subunit I, cytochrome *b*, and ATP synthetase. Five of the clones represented overlapping sequences of the identified mRNAs.

To augment the amount of sequence information available on the 14 remaining cDNAs, the clones were extended using 5′- and/or 3′-RACE methodology. This experimental approach facilitated the identity of 11 additional clones. Eight encoded mRNAs that were previously identified. Three new clones where identified: selenoprotein W, low density lipoprotein (LDL) receptor adaptor protein, and cytochrome *c* oxidase subunit Vb. We were unable to extend the sequence of the three remaining clones. In total, the differential mRNA experiment led to the identification of 27 nuclear-encoded mRNAs (Table 1) and an additional four mitochondrial mRNAs.

Nerve terminals contain a heterogeneous mRNA population

To test the hypothesis that the presynaptic nerve terminal also contains mRNAs, we employed a synaptosomal preparation obtained from the squid optic lobe. This preparation is comprised predominately of the

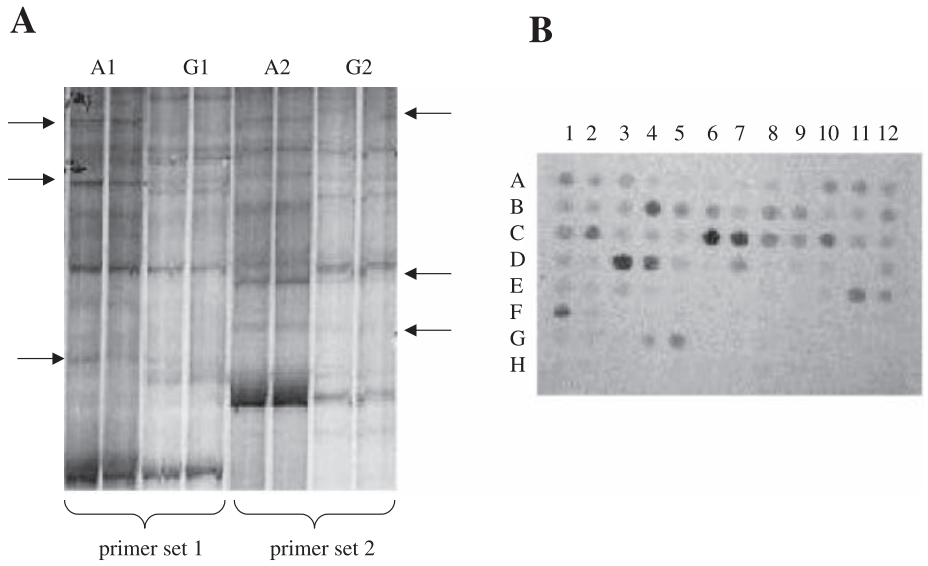

FIG. 1. Experimental design employed in the identification of axonal mRNAs. (A) Differential mRNA display. Total RNA from the giant axon (lanes A1, A2) and the parental cell soma located in the GFL (lanes G1, G2) were reverse-transcribed into cDNA and fragments amplified by PCR, using a set of random oligonucleotides as primers as described in Material and methods. Amplicons from the axon and GFL were then displayed on polyacrylamide-urea sequencing gels. Arrows indicate amplicons preferentially expressed in the axon (lanes A1 and A2) that were selected for further analysis. (B) Cross-species hybridization analysis. Selected axonal cDNAs ($n = 150$) were affixed to nylon filter supports and were hybridized to radiolabelled rat brain cDNAs. A human β-actin cDNA served as an internal positive control (Clone G5). A subset of clones that manifested evolutionary sequence conservation ($n = 50$) was selected for nucleic sequencing. The identity of these clones was subsequently established by comparative sequence analysis and the results are summarized in Table 1.

TABLE 1. Comparative sequence analysis of squid giant axon mRNAs (highest Blast X results)

mRNA compared	Species	GI	*E*-value	Identity (%)	Similarity (%)
β-Actin	Zebra mussel	3421451	0.0	97	100
Cytochrome *c* oxidase subunit Vb	*Drosophila*	32815893	$2 \times e^{-17}$	51	71
Cytochrome *c* oxidase subunit XVII	King cobra	10442710	$7 \times e^{-07}$	62	78
Coenzyme Q biosynthetic enzyme	Mouse	5921833	$3 \times e^{-46}$	63	83
Dihydrolipoamide dehydrogenase	*Trypanosoma cruzi*	6166121	$2 \times e^{-07}$	68	78
Elongation factor 2	Tiger centipede	13111512	$2 \times e^{-99}$	67	79
Enolase	Squid	1911572	$1 \times e^{0.0}$	100	100
HSP70	Clam	38683404	$4 \times e^{-37}$	91	96
Kinesin	Squid	469682	$1 \times e^{0.0}$	100	100
LDL receptor adaptor protein	Mouse	21704092	$4 \times e^{-25}$	53	82
Nucleoside diphosphate kinase	Human	37693993	$3 \times e^{-17}$	62	85
Protein phosphatase 2C	Rat	38541341	$2 \times e^{-32}$	54	76
Ribosomal proteins					
S5	Pacific oyster	40643036	$7 \times e^{-69}$	94	98
S8	*Xenopus laevis*	3248427	$3 \times e^{-48}$	75	84
S25	Human	4506707	$7 \times e^{-25}$	77	87
S27A	Rat	34872524	$4 \times e^{-23}$	80	87
L7	Army worm	18253049	$2 \times e^{0}$	82	94
L7A	Puffer fish fugu	6094094	$1 \times e^{-100}$	68	81
L8	Pacific oyster	40643032	$4 \times e^{0}$	68	80
L9	Rat	27669155	$2 \times e^{-14}$	54	59
L27A	Rainbow trout	14716988	$3 \times e^{-41}$	69	80
Selenoprotein W	Zebrafish	30231222	$2 \times e^{-20}$	58	75
β-Spectrin	*C. elegans*	17569483	$3 \times e^{-36}$	82	90
α-Tubulin	Leech	1527172	$8 \times e^{-78}$	93	94
β-Tubulin	Sea urchin	135489	$1 \times e^{0.0}$	93	95
Ubiquitin C	Zebrafish	29612653	$6 \times e^{-25}$	97	97

GI, GenBank sequence identification number. *E*-value is the number of hits expected by chance when searching a database.

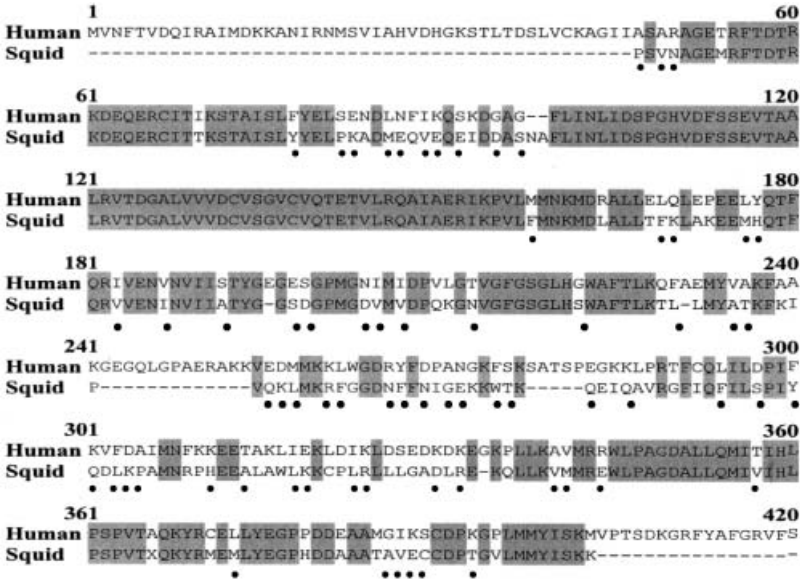

FIG. 2. Comparison of the partial amino acid sequence of squid and human elongation factor-2. Shaded regions signify sequence identity. Closed circles indicate a conservative amino acid substitution. Overall, the squid sequence manifests 58% sequence identity with the human ortholog. The complete primary sequence of human elongation factor-2 protein is comprised of 858 amino acids.

large nerve terminals (1–3 μm diameter) of the retinal photoreceptor neurons (Crispino *et al*., 1997). As the optic lobe is comprised mainly of axon–axonal synapses, there are few dendritic elements in the preparation. In this experiment, total RNA was isolated from the synaptosomal fraction and the presence of mRNA determined by RT-PCR, using gene-specific primer sets for β-actin, β-tubulin, synapsin, neurofilament protein, and the sodium channel. The results of this experiment are shown in Fig. 3. Amplicons for each of these mRNAs, save tubulin (see below) were generated from total cytosolic and synaptosomal RNA, indicating that mRNAs for these proteins were present in both the perikaryon and nerve terminal. Interestingly, amplicons for tubulin mRNA were not detected in synaptosomal RNA, although they were highly abundant in the cytosolic fraction. This finding suggests that not all mRNAs located in the axon are present in the nerve terminal, and argues against the possibility that these data derive from cytoplasmic contamination of the synaptosomal fraction.

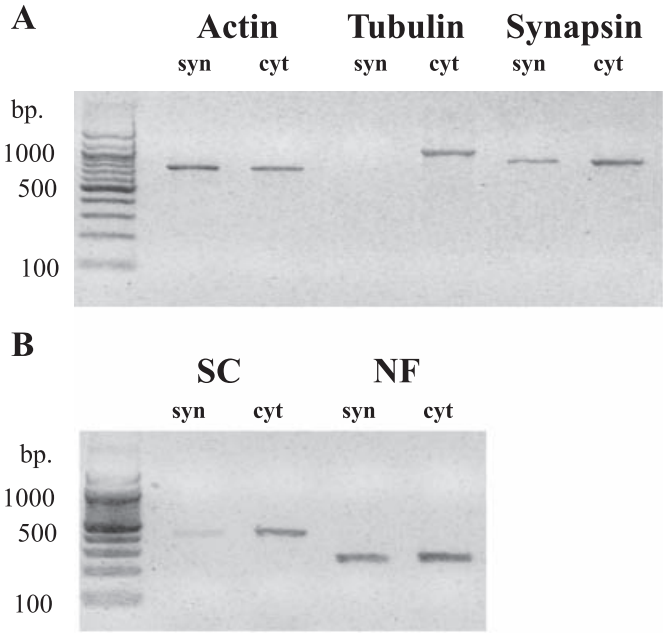

FIG. 3. Synaptosomes contain a diverse population of mRNAs. RT-PCR analysis was performed on total RNA from the synaptosomal (syn) and cytosolic (cyt) fractions from the squid optic lobe. SC, sodium channel; NF, neurofilament protein.

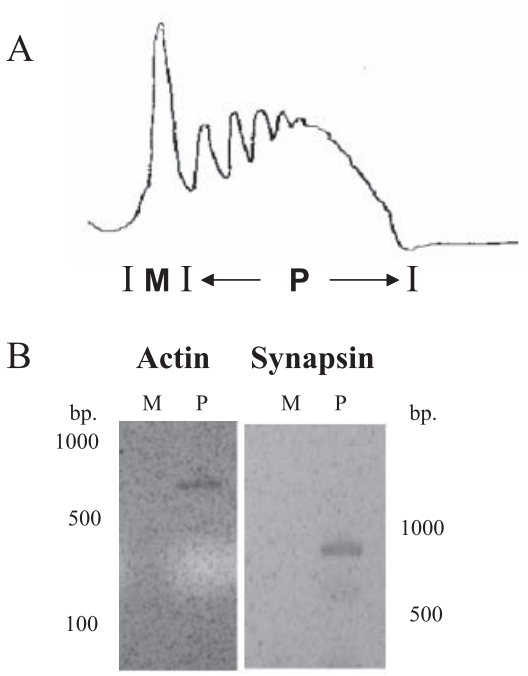

FIG. 4. Messenger RNAs are actively translated in the synaptosome. Polysomes were prepared from squid optic lobe synaptosomes as described in Materials and methods. (A) Polysomes were displayed on linear sucrose density gradients and the gradients divided into monosome (M) and polysome (P) fractions. UV absorbance of RNA was monitored continuously at 254 nm (B) RT-PCR analysis of monosome and polysome fractions using gene-specific primer sets for β-actin, and synapsin. bp, base-pairs.

To determine if mRNAs were being translated in the nerve terminal, polysomes were isolated from synaptosomes and were subsequently displayed on linear sucrose density gradients.

RT-PCR, using gene-specific primers for β-actin and synapsin, was conducted on the RNA isolated from both the monosome and polysome fractions of the gradients. As shown in Fig. 4, β-actin and synapsin sequences were readily detected in the polysome fraction, providing strong evidence that these mRNAs were actively translated in the nerve terminal.

Nerve terminals translate a heterologous reporter mRNA

To further explore the possibility that the presynaptic nerve terminal of the squid photoreceptor neuron contains a local protein synthetic system we transfected the synaptosomal preparation with a GFP reporter mRNA. The immunohistochemical visualization of synapsin was used to confirm the presynaptic terminal localization of the GFP translation product. The transfection of the antisense GFP RNA served as a negative control. In these experiments, GFP translation products were readily observed in synaptosomes 30–60 min after transfection with GFP mRNA (Fig. 5, T7 promoter), whereas no signal was obtained after transfection with the antisense RNA (Fig. 5, T3 promoter). Importantly, the GFP rhodamine signal was coincident with the FITC signal obtained using an antibody to synapsin, a protein marker for the nerve terminal. These data clearly establish that the presynaptic nerve terminals of squid photoreceptor neurons are capable of synthesizing protein.

Discussion

To gain insight into the function(s) of the local protein synthetic system, we initiated a characterization of the constituents of the mRNA populations present in the axon and nerve terminal, using differential mRNA display as an unbiased experimental approach. Among the mRNAs identified in this report, several had been isolated previously from a random screening of a cDNA library prepared from the squid giant axon. These mRNAs included β-actin, β-tubulin, kinesin heavy chain and enolase, and the axonal localization of each mRNA firmly established by *in situ* hybridization histochemistry (Kaplan *et al*., 1992; Gioio *et al*., 1994; Chun *et al*., 1995, 1996). This duplication likely arises from the relatively high abundance of these mRNAs in the axon, as judged by quantitative RT-PCR analysis (Chun *et al*., 1996).

One unexpected finding of this study was the axonal presence of several mRNAs encoding ribosomal proteins. Recently, Martin and colleagues have also reported the axonal localization of several ribosomal protein mRNAs in *Aplysia* sensory neurons (Moccia *et al*., 2003). Although ribosomal proteins are known to manifest multiple functions, the large number of these mRNAs in the axon renders it unlikely that they subserve extra-ribosomal functions. As noted by Moccia *et al*. (2003), several of the ribosomal proteins identified are located superficially on both ribosomal subunits and hence could participate in ribosomal subunit assembly and/or the mediation of mRNA–ribosomal interactions. These surface proteins could also manifest a shorter half-life than those located within the interior of the organelle and thus might require local synthesis to maintain the translational activity of the ribosome.

The localization of EF-2 mRNA in the axon and nerve terminal raises an intriguing possibility regarding the regulation of local protein synthesis. The activity of this elongation factor, as well as IF-2, is inhibited by calcium-dependent phosphorylation that ultimately results in the inhibition of protein synthesis (Nygard *et al*., 1991; Alcazar *et al*., 1997). Previously, we reported that the protein synthetic activity of the optic lobe synaptosome preparation was markedly affected by

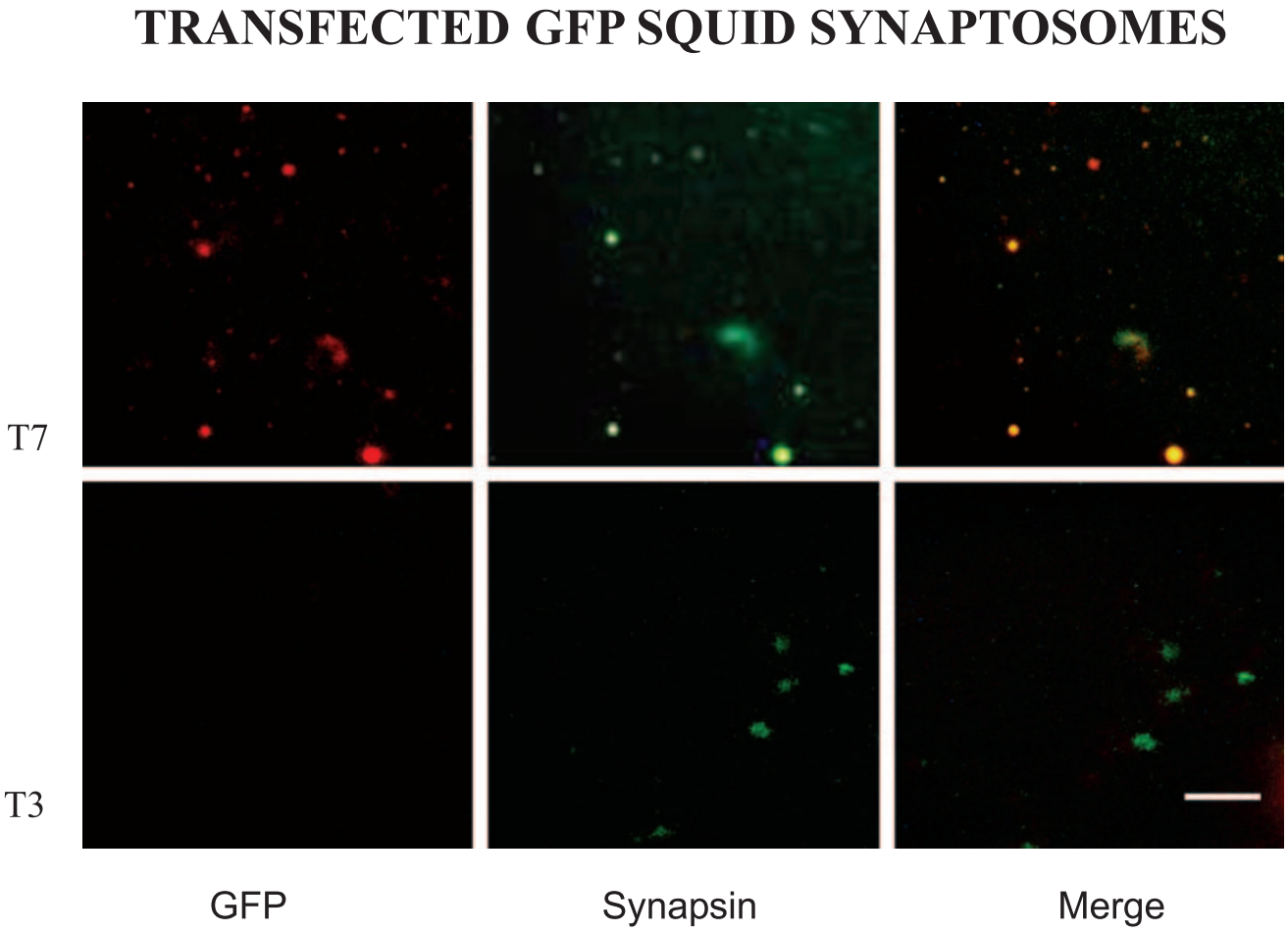

FIG. 5. Synaptosomes translate a GFP reporter mRNA. GFP mRNA (T7) was introduced into synaptosomes *in vitro*, using a lipid-based transfection methodology and GFP synthesis monitored by immunofluorescence using a rhodamine-conjugated antibody (see Materials and methods). An antisense GFP RNA (T3) was used as a negative control Synapsin was employed as a marker protein for the presynaptic nerve terminal and was visualized by immunohistochemistry using a FITC-labelled secondary antibody. Scale bar, 10 μm.

cytosolic calcium levels (Benech *et al*., 1999). Hence, the activity-dependent, post-translational modification of either of these factors could play an important role in the regulation of local protein synthesis.

Previously, we reported the surprising finding that the squid giant axon and photoreceptor nerve terminal contained several nuclear-encoded mRNAs for mitochondrial proteins and molecular chaperones (e.g. HSP70) that might function to facilitate the transport of cytosolically synthesized proteins into the mitochondrion (Gioio *et al*., 2001). Results of *in vitro* radiolabelling experiments conducted in the optic lobe synaptosomal preparation clearly indicated that a significant number of nuclear-encoded mitochondrial-associated proteins were synthesized locally, and that 20–25% of the protein synthesized in the nerve terminal was destined for this organelle (Gioio *et al*., 2001). The isolation of a fourth such mRNA, cytochrome oxidase subunit Vb, is consistent with these findings. These observations call to attention the intimacy of the relationship that exits between the nerve terminal and its resident mitochondria.

In view of the fact that the axonal mRNA population has heretofore been little studied, it is not surprising to encounter several novel mRNAs. Interestingly, our initial biochemical and immunohistochemical findings on the novel pA6 mRNA indicates that it encodes a small, highly basic, mitochondrial-associated protein that is preferentially expressed in neurons of both the squid and rodent nervous systems (Wen *et al*., 2002). Preliminary data acquired on pA134 suggests that this mRNA codes for a 34 kDa phosphotyrosine binding protein (PTB) that manifest approximately 50% sequence identity to the human low density lipoprotein receptor adaptor protein (Garcia *et al*., 2001). This observation suggests that pA134 might play a role in receptor mediated endocytosis and/or vesicular transport/recycling. Last, the mRNA (pA127) encodes an 87 amino-acid protein that shares significant sequence similarity (75%) to a selenoprotein, a family of recently discovered proteins that are thought to be involved in protection from oxidative damage (reviewed in Chen & Berry, 2003). The expression and function of these novel mRNAs and their cognate protein are now under investigation.

Results of a recent independent proteomics study of local protein synthesis in squid brain synaptosomes revealed the *de novo* synthesis of approximately 80 different proteins, as judged by two-dimensional polyacrylamide gel electrophoresis (Jimenez *et al*., 2002). Consistent with the findings obtained at the mRNA level, mass spectrometric analysis of the newly synthesized protein established the presence of several cytosolic enzymes, cytoskeletal proteins, molecular chaperones, and nuclear-encoded mitochondrial proteins. In addition, the synthesis of a number of novel proteins was detected. As shown in this report, this synaptosomal preparation is also capable of translating a transfected GFP mRNA, used here as a heterologous reporter mRNA. The immunohistochemical colocalization of GFP with synapsin

TABLE 2. mRNAs identified in invertebrate axons

mRNA	References
Cytoskeletal proteins	
β−Actin	Kaplan *et al*. (1992)
α- & β-Tubulin	Kaplan *et al*. (1992) and Moccia *et al*. (2003)
β-Spectrin	This report
β-Thymosin	Moccia *et al*. (2003)
Neurofilament proteins	Giuditta *et al*. (1991)
Molecular motors	
Kinesin	Gioio *et al*. (1994)
MAP HI	Chun *et al*. (1996)
Ribosome associated proteins	
S5, S6, S15, S16, S19, S29	Moccia *et al*. (2003) and this report
L7A, L8, L9, L11, L18	Moccia *et al*. (2003) and this report
L22, L31, L36, L37	Moccia *et al*. (2003) and this report
Translation factors	
EF 1α	Moccia *et al*. (2003)
EF 2	This report
CPED	Moccia *et al*. (2003)
Nuclear-encoded mitochondrial protein	
Cytochrome oxidase assembly protein COX17	Gioio *et al*. (2001)
Cytochrome oxidase subunit Vb	This report
Ubiquinone biosynthesis protein CoQ7	Gioio *et al*. (2001)
Dihydrolipoamide dehydrogenase	Gioio *et al*. (2001)
Neuropeptides	
Sensorin	Schacher *et al*. (1999) and Moccia *et al*. (2003)
Caudodorsal cell hormone	Dirks *et al*. (1993)
APG-wamide	van Minnen (1994)
Other Proteins	
Enolase	Chun *et al*. (1995)
Fructose PTS enzyme II	This report
Heat shock protein 70	Gioio *et al*. (2001)
LDL receptor adapter protein	This report
Nucleoside diphosphate kinase	This report
Selenoprotein W	This report
Syntaxin	Hu *et al*. (2003)
Ubiquitin	Moccia *et al*. (2003) and this report

establishes that the reporter mRNA was translated in presynaptic nerve terminals *per se*. Taken together, the results of these studies demonstrate that the presynaptic nerve terminals of squid photoreceptor neurons synthesize a wide variety of proteins involved in synaptic function, and establish that the squid optic lobe synaptosomal preparation will serve as a useful model system in which to investigate the regulation of local protein synthesis and provide a rich resource for the identification of novel gene products.

To date, approximately 40 different mRNAs have been identified in invertebrate axons and nerve terminals (Table 2). Although this value represents only 15–25% of the mRNAs predicted to be present in these distal structural and functional domains (Perrone Capano *et al*., 1987; Moccia *et al*., 2003), there is now sufficient information to formulate several working hypotheses regarding the function of the local protein synthetic system in the adult nervous system. As shown in Table 2, these mRNAs could be grouped into several broad functional categories. This classification would suggest that local protein synthesis could subserve the maintenance and/or remodelling of the cytoarchitecture of the axon and terminal, maintain the local axon transport and mRNA translation systems, as well as contribute to the viability and function of the local energy generating system and the maintenance of local calcium homeostasis. Interestingly, the injection of isolated *L. stagnalis* axons with mRNA encoding the conopression receptor resulted in the expression of active receptor on the axonal plasma membrane (Spencer *et al*., 2000). This finding clearly demonstrates that invertebrate axons are also capable of the synthesis and post-translational modification and membrane insertion of cell surface proteins, and raises the distinct possibility that the local protein synthesis plays an important role in the expression of neuronal cell surface receptors and transporters. In this regard, it is noteworthy, that the activity-dependent synthesis of glutamate receptors from transfected mRNAs has been recently demonstrated in the dendrites of cultured hippocampal neurons (Kacharmina *et al*., 2000; Ju *et al*., 2004). In the adult nervous system, the local protein synthetic system might prove especially important in large asymmetric neurons, where the cell soma is far removed from the terminal, or alternatively in neurons that manifest collateral axons or a large highly ramified terminal aborization. It is noted, that although the results in this communication derive exclusively from invertebrate motor and sensory systems, similar findings have now been reported in vertebrate nervous systems (Koenig, 1991; Olink-Coux & Hollenbeck, 1996; Bassell *et al*., 1998; Eng *et al*., 1999; Koenig *et al*., 2000; Zheng *et al*., 2001; Lee & Hollenbeck, 2003), and, hence, these reports also serve to highlight the general applicability of the data generated from invertebrate model systems.

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Abbreviations

EF-2, elongation factor 2; GFL, giant fibre lobe; GFP, green flourescent protein; HRP, horse radish peroxidase.

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