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Medium weight neurofilament mRNA in goldfish Mauthner axoplasm

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Abstract

Although axons are generally considered to lack the ability to synthesize proteins, the Mauthner axon (M-axon) of the goldfish has been reported to contain some of the basic components of the translational machinery, such as transfer RNA (tRNA), ribosomal RNA (rRNA), and ribosomes. To determine if the M-axon also contains mRNA, we isolated samples of M-axoplasm free of glial contamination as demonstrated by the absence of glial-specific mRNA and protein. Reverse transcription-polymerase chain reaction (RT-PCR) of M-axoplasmic cDNA in the presence of primers for the goldfish medium-weight neurofilament (NF-M) gene produced a single product of the expected length for RT-PCR amplification of goldfish NF-M mRNA. This mRNA might direct protein synthesis of NF-M within the M-axoplasm.

Keywords

Axoplasmic protein synthesis; Goldfish Mauthner axon; mRNA; Neurofilament protein

The nerve axon is generally considered to depend entirely on its cell body for trophic support such as synthesis of proteins necessary to maintain axonal structure and function. This assumption is supported by data showing that a distal axonal segment severed from its cell body in mammals typically degenerates within hours to days [10]. However, when axons are severed from their cell bodies in invertebrates [2] or lower vertebrates such as the goldfish [18], the distal axonal segment often survives for months to years. Proteins in an axon isolated from its cell body could be maintained only through some combination of (1) slow turnover of existing proteins; (2) local transfer of proteins from adjacent cells such as glia; and/or (3) local axoplasmic protein synthesis [2].

Although often regarded as a particularly unlikely mechanism to maintain axonal proteins [2, 16], axoplasmic protein synthesis has been reported for squid giant axons [7] and goldfish Mauthner axons (M-axons) [14]. The M-axon is a particularly advantageous preparation because more basic components of the translational machinery (transfer RNA (tRNA), ribosomal RNA (rRNA), and ribosomes) have been identified in the M-axon [13–15] compared to any other vertebrate axon. However, no direct evidence has been published for any mRNAs that could function as templates for protein synthesis in M-axoplasm, although mRNAs have been reported in other vertebrate axons [9,19] for which the presence of translational machinery

has not yet been examined. In this study, we use reverse transcription-polymerase chain reaction (RT-PCR) to probe for the presence of neurofilament (NF-M) in the M-axon.

In addition to published data on the presence of all basic components of the translational machinery, other benefits of the goldfish M-axon as an experimental system to examine protein synthetic capabilities in a vertebrate axon include (1) its long length (5–8 cm) and large diameter (50–80 μm) provide a large amount of axoplasm per cell for biochemical analysis; (2) its high axonal viscosity and large axonal diameter permit a clean separation of its axoplasm (M-axoplasm) from its surrounding glial sheath (M-sheath) [14,18]; and (3) the availability of goldfish cDNA sequences from distinctly neuronal (NF-M) mRNA transcripts and distinctly glial mRNA transcripts (glial filament acidic protein; GFAP) permits sensitive PCR-based contamination controls to verify clean axonal isolation.

Goldfish (*Carassius auratus*) brain, M-sheath, and M-axoplasm were isolated as previously described [14,18]. M-axoplasm was rinsed in several changes of ice-cold calcium-free saline and then lysed by vortexing in an extraction buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroyl-sarkosinate, 0.1 M β -mercaptoethanol, and 20 $\mu\text{g/ml}$ 5S r-RNA from *Escherichia coli* (Boehringer Mannheim) added as a carrier. M-axoplasm from 20 M-axons was pooled, and total RNA was isolated by the method of Chomczynski and Sacchi [4]. First-strand cDNA was synthesized from RNA according to standard protocols [21].

Amplification of one-seventh of the total cDNA product of M-axoplasm cDNA or one-ten thousandth of the total brain cDNA from goldfish brain was performed in 50 μl of a solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2 mM MgCl_2 , 0.1% Triton X-100, 0.4 mM deoxynucleotide triphosphates, 10 μCi [α - ^{32}P]dATP (DuPont), 200 ng each of forward and reverse primers for either NF-M or GFAP, and 2.5 units of *Taq* DNA polymerase. The reaction was amplified for 35 cycles, each consisting of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. PCR products were analyzed on a 6% polyacrylamide gel containing 8.3 M urea. The position of the PCR products was detected by autoradiography with an intensifying screen for 1 h at -80°C. False positives due to amplification of genomic DNA were avoided by designing the primer pairs so that each primer was derived from a separate exon (Fig. 1). All PCR reactions were repeated at least twice using new RNA preparations obtained from separate collections of goldfish brain and M-axoplasm to verify the reproducibility of these experiments.

PCR amplification of goldfish brain cDNA or goldfish M-axoplasm cDNA with forward and reverse NF-M primers yielded a single product of approximately 387 nucleotides (Fig. 2A, lanes 1 and 2), the predicted product for RT-PCR amplification of goldfish NF-M mRNA (Fig. 1A). Amplification of the reagents for NF-M PCR without the inclusion of cDNA derived from goldfish tissue failed to yield a detectable PCR product (Fig. 2A, lane 3). These data indicated that NF-M mRNA was present in M-axoplasmic samples.

To verify that the M-axoplasmic samples used for NF-M amplification were free of glial contamination, we attempted to amplify GFAP transcripts (a glial-specific transcript) from the same M-axoplasmic cDNA samples used for PCR amplification of NF-M transcripts. Total cDNA prepared from adult goldfish brain and M-axoplasm was subjected to amplification by PCR in the presence of primers corresponding to nucleotides 648–672 and 975–999 of goldfish GFAP cDNA (Fig. 1B). PCR amplification of goldfish brain cDNA yielded a single product of approximately 352 nucleotides (Fig. 2B, lane 1), the predicted product for RT-PCR amplification of goldfish GFAP mRNA (Fig. 1B). Both the amplification of M-axoplasmic cDNA and the amplification of the reagents for GFAP PCR amplification without the inclusion of goldfish-derived cDNA failed to yield any detectable PCR products (Fig. 2B, lanes 2 and 3). These data suggest that glial mRNA does not contaminate the M-axoplasmic preparation.

The possibility that M-axoplasm was contaminated with surrounding glial tissue was further investigated by comparing silver stains or immunoblots of axoplasmic and sheath proteins. Gels containing samples of goldfish M-axoplasm and M-sheath were either silver-stained or analyzed via immunoblotting [18] with monoclonal antibodies directed against GFAP, a glial-specific protein [10]. Sodium dodecyl sulfate (SDS) gels of M-axoplasm produced six prominent silver stained bands at 235, 145, 123, 105, 80, and 60 kDa (Fig. 3, lane 1, lines to left of figure). These bands have been identified as neurofilament proteins according to their biochemical and immunological characteristics [18]. SDS gels of M-sheath produced more silver-stained bands with a much broader range of molecular weights than the silver-staining bands for M-axoplasm (Fig. 3, lane 1). Samples of M-sheath also contained an intensely silver-staining band at 50 kDa which corresponded to the molecular weight of GFAP (arrow to the right of lane 4 in Fig. 3). The intensely-staining GFAP-containing band did not appear in silver-stained gels of M-axoplasm (compare lanes 1 and 4 in Fig. 3). Anti-GFAP immunoblotting of M-axoplasm and M-sheath followed by enhanced chemiluminescence produced a GFAP-reactive band for M-sheath at the expected molecular weight for GFAP (Fig. 3, lane 3), but no detectable band for M-axoplasm (Fig. 3, lane 2).

Taken together, these data show that NF-M mRNA in our samples of M-axoplasm does not result from contamination by surrounding tissue, since macromolecules such as GFAP mRNA and GFAP protein from the immediately-adjacent glial tissues are not detected in our samples of M-axoplasm. These data suggest that the NF-M RT-PCR product observed in M-axoplasmic samples is due to specific amplification of NF-M mRNA in the M-axoplasm.

NF-M transcripts in M-axoplasm might serve several functional roles. First, NF-M might act as a reserve to supplement similar mRNA transcripts in the cell body, as proposed for tyrosine hydroxylase mRNA in rat hypothalamic axons [22]. Second, NF-M transcripts might be transferred to adjacent glia where neurofilament proteins could be synthesized on rough ER followed by glia-to-axon transfer of NF-M protein. Third, NF-M transcripts in M-axoplasm might direct the axoplasmic synthesis of NF-M.

Although controversial [2,16], local axoplasmic protein synthesis would have several advantages. First, since one mRNA can be translated many times, transport of mRNA from the cell body to the axonal compartment followed by synthesis of proteins within the axon would be a more efficient mechanism to supply the axon with protein than transport of each protein from the cell body to the axon. Second, since slowly-transported axonal proteins are estimated to have half-lives on the order of days to weeks [20,23] and the rate of axonal transport of cytoskeletal proteins such as actin, tubulin, and the neurofilament proteins is 0.25–3 mm/day [3], significant protein degradation would be expected to occur during transport, especially for long axons. These degraded cytoskeletal proteins might be supplemented by local axoplasmic protein synthesis. Third, axoplasmic synthesis of cytoskeletal proteins such as NF-M could play an important role in the construction and maintenance of the highly polar neuronal cytoskeleton. For example, the prevention of β -actin mRNA localization to the cell periphery of chicken embryonic fibroblasts results in the disorganization of the actin cytoskeleton, suggesting that the maintenance of cell polarity requires localized protein synthesis of this cytoskeletal element [11].

In conclusion, the discovery of NF-M mRNA within the goldfish M-axon increases the number of known axonally localized mRNAs, including oxytocin mRNA in the rat hypothalamo-neurohypophyseal tract [9], caudodorsal cell hormone mRNA in the central nervous system of the mollusc *Lymnaea stagnalis* [5], kinesin mRNA in the squid giant axon [6], and arginine vasopressin precursor mRNA in rat hypothalamo magnocellular neurons [19]. The presence of mRNA in the M-axon is particularly significant given previous reports of tRNA, rRNA, polyribosomes, and protein synthesis in the M-axon [13-15]. If the presence of mRNA in the

M-axon directs local protein synthesis, and if these mRNAs have a long half-life as reported for mRNAs in the giant unicellular alga *Acetabularia* [12], then local synthesis of axonal proteins might provide part of the explanation for the experimental observation that a severed M-axon can survive for many months in the absence of its cell body [18].

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A. Goldfish NF-M Gene

B. GFAP Gene

Fig. 1.

Location and sequence of primers used in PCR amplifications. (A) Goldfish NF-M gene. The horizontal lines indicate location of introns whereas the boxes indicate the location of exons for the goldfish NF-M gene. The location of the sense and antisense primers used in PCR amplifications are denoted by forward and reverse half-arrows. Sense and antisense primers correspond to nucleotides 1716–1740 and 3119–3243, respectively, of the goldfish NF-M gene [8]. (B) GFAP gene. Intron/exon structure and primer designations are as described in (A). The location and size of introns for the goldfish GFAP gene were inferred by comparing the sequence of goldfish GFAP eDNA (Glasgow and Schechter, unpublished; Genbank Accession Number L23876) with the published sequence for the mouse GFAP gene [1]. The application of this method to compare the goldfish NF-M gene [8] with the mouse NF-M gene [17] showed that intron location, but not intron size, was conserved between goldfish and mouse. Sense and antisense primers correspond to nucleotides 648–672 and 975–999, respectively, of goldfish GFAP eDNA (Glasgow and Schechter, unpublished; Genbank Accession Number L23876).

Fig. 2.

(A) Products of PCR amplification of NF-M mRNA in goldfish brain and M-axoplasm. Total eDNA prepared from adult brain (lane 1) and M-axoplasm (lane 2) was subjected to 35 cycles of PCR amplification using forward and reverse NF-M primers. Lane 3 contains the amplification product of the reagents for NF-M PCR amplification without the inclusion of eDNA derived from goldfish tissue. Each lane contains 20% of a total PCR amplification. The arrow labeled NF-M indicates the theoretical location of the PCR product for NF-M mRNA at 387 nucleotides. The numbers to the left of (A,B) indicate the nucleotide length of *Hpa*II cut pUC19 marker DNA. (B) Products of PCR amplification of GFAP mRNA in goldfish brain and M-axoplasm. The same brain and M-axoplasmic eDNA samples shown in (A) were subjected to 35 cycles of PCR amplification using forward and reverse GFAP primers. Lane 3 contains the amplification product of the reagents for GFAP PCR amplification without the inclusion of eDNA derived from goldfish tissue. PCR products were analyzed as in (A). The arrow labeled GFAP indicates the theoretical location of the PCR product for GFAP mRNA at 352 nucleotides.

Fig. 3.

Silver-stained gel and anti-GFAP immunoblot of M-axoplasm and M-sheath. Goldfish M-axoplasm and M-sheath were electrophoretically analyzed on 10 to 15% gradient denaturing polyacrylamide gels. The gels were either silver-stained or the proteins were transferred to a nitrocellulose filter and probed with monoclonal antibodies directed against GFAP. Lanes 1 and 4 contain silver-stained M-axoplasmic and M-sheath proteins, respectively. Lanes 2 and 3 contain anti-GFAP immunoblots of M-axoplasm and M-sheath, respectively. The lines to the left of the figure indicate the location of neurofilament proteins in M-axoplasm at 235, 145, 123, 105, 80, and 60 kDa. The arrow to the right of the figure indicates the location of GFAP in M-sheath at 50 kDa.