Aldolases A and C Are Ribonucleolytic Components of a Neuronal Complex That Regulates the Stability of the Light-Neurofilament mRNA

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A 68 nucleotide segment of the light neurofilament (NF-L) mRNA, spanning the translation termination signal, participates in regulating the stability of the transcript in vivo. Aldolases A and C, but not B, interact specifically with this segment of the transcript in vitro. Aldolases A and C are glycolytic enzymes expressed in neural cells, and their mRNA binding activity represents a novel function of these isozymes. This unsuspected new activity was first uncovered by Northwestern blotting of a brainstem/spinal cord cDNA library. It was confirmed by two-dimensional fractionation of mouse brain cytosol followed by Northwestern hybridization and protein sequencing. Both neuronal aldolases interact specifically with the NF-L but not the heavy neurofilament mRNA, and their binding to the transcript excludes the poly(A)-binding protein (PABP) from the complex. Constitutive ectopic expression of aldolases A and C accelerates the decay of a neurofilament transgene (NF-L) driven by a tetracycline inducible system. In contrast, mutant transgenes lacking mRNA sequence for aldolase binding are stabilized. Our findings strongly suggest that aldolases A and C are regulatory components of a light neurofilament mRNA complex that modulates the stability of NF-L mRNA. This modulation likely involves endonucleolytic cleavage and a competing interaction with the PABP. Interactions of aldolases A and C in NF-L expression may be linked to regulatory pathways that maintain the highly asymmetrical form and function of large neurons.

Key words: neuronal aldolases; neurofilaments; RNA binding; posttranscriptional regulation; endonucleolytic cleavage; distributive exonuclease; poly(A)-binding protein; PABP

Introduction

Diversity in protein evolution is generally believed to arise from mutations in reduplicated genes (Brosius, 2003). Occasionally, bifunctional activities are discovered in a single gene product. Alternatively, they may reflect the derivation of multiple functions as a result of changes in subcellular localization, conformational state, concentration of interactive molecules, or cell specificity (Wilkinson and Shyu, 2001; Jeffery, 2003). The latter possibility is exemplified by enzymes in the glycolytic pathway. Whereas glycolytic enzymes have evolved as a highly conserved, universal set of enzymes for converting monosaccharides into energy, they also reveal some vestiges of an ancient past. It has been known that enzymes display RNA-binding properties, suggesting a possible linkage to a primordial RNA-binding world (Hentze, 1994). In some instances, these RNA-binding properties may have evolved as moonlight activities, such as the one exemplified by the role of aconitase in regulating iron metabolism in the cell (Kennedy et al., 1992; Hentze and Kuhn, 1996) or the RNA binding activities of the nicotinamide adenine dinucleotide-dependent dehydrogenases (Sioud and Jespersen, 1996; Nagy et al., 2000; Pioli et al., 2002). The nuclear form of glyceraldehyde-3-phosphate dehydrogenase binds directly to Oct1 and is part of a complex that links the histone H2B transcriptional machinery to the cell cycle and metabolic cell status (Zheng et al., 2003).

The markedly asymmetrical neuronal shape is established and maintained by the synthesis, assembly, and translocation of cytoskeletal proteins from the neuronal perikarya into neuritic processes. The extraordinary size of large axons in subsets of neurons with dimensions >20,000-fold greater than the size of the perikarya is maintained by vast numbers of neurofilaments (NFs) that fill the axon and undergo a steady slow proximodistal axonal translocation. NFs are heteropolymers in vivo and require the presence of the light subunit (NF-L) for assembly (Ching and Liem, 1993; Lee et al., 1993), thereby conferring a dominant role to the regulation of NF-L subunit expression. Deletion of NF-L does not lead to an obvious phenotype (Zhu et al., 1998), but overexpression of a wild-type (wt) NF-L transgene (Xu et al., 1993) or low-level expression of a mutant NF-L transgene (Lee et
transferred to polyvinylidene difluoride membranes, and replicate sham Biosciences) and the second dimension SDS-PAGE using a Hoefer brain proteins was performed by isoelectric focusing using an IPGphor protocols. Brain and liver as well as cell line extracts were prepared as established by the University of Pennsylvania Animal Regulatory Affairs. Laboratories, Wilmington, MA) were used following the guidelines established by the University of Pennsylvania Animal Regulatory Affairs. Cell lines were purchased from American Type Culture Collection (Ma- nassas, VA) and cultured following American Type Culture Collection protocols. Brain and liver as well as cell line extracts were prepared as described previously (Cañete-Soler et al., 1998b).

Northwestern screening of a brainstem/spinal cord cDNA library. The Northwestern screening of the brainstem/spinal cord cDNA library was performed as described previously (Cañete-Soler et al., 2001).

Two-dimensional PAGE and matrix-assisted laser desorption mass spectrometry. The two-dimensional (2D) fractionation of 50 μg of mouse brain proteins was performed by isoelectric focusing using an IPGphor isoelectric focusing system and precast immobile IPG strips (Amersham Biosciences) and the second dimension SDS-PAGE using a Hoefer SE600 vertical system (Hoefer Scientific, San Francisco, CA). All of the procedures followed the instructions of the manufacturer. Proteins were transferred to polyvinylidene difluoride membranes, and replicate samples were hybridized to internally labeled NF-L 68 RNA or nonspecific SK +70 RNA or end-labeled horseradish peroxidase (poly C) to selectively identify bona fide interactions. Proteins aligned with specific neurofilament mRNA hybridization spots were excised and submitted to the protein facility at the Wistar Institute for matrix-assisted laser desorption mass spectrometry (MALDI-MS) and Edman sequencing (Speicher and Reim, 1977; Speicher et al., 2000)

Expression and purification of recombinant aldolases. Full-length sequences of aldolases A and C were obtained by PCR of a mouse spinal cord cDNA library using specific primers followed by in-frame subcloning into appropriate restriction sites of pGEX-6 vectors. All constructs were fully sequenced by the DNA Sequencing Facility at the University of Pennsylvania School of Medicine (Philadelphia, PA). Glutathione S-transferase (GST)/aldolase fusion proteins were expressed and purified following standard procedures, and the GST moiety was removed by PreScission protease (Amersham Biosciences) followed by three rounds of purification using glutathione Sepharose 4B columns according to the protocols of the manufacturer. Purified aldolases were dialyzed against buffers for downstream applications and aliquoted and kept at −70°C.

Electrophoretic mobility shift, Northwestern assay, and Western blot assay. Electrophoretic mobility shift assays were performed as described previously (Chen et al., 1992) with minor modifications. Briefly, 10–75 μg of cytoplasmic extracts (brain or liver) or 0–0.250 μg purified recombinant proteins was incubated with 1 ng of gel-purified radiolabeled NF-L wt or NF-L mutant variants for 5 min at 37°C, followed by incubation with RNase T1 for 10 min at 37°C. Reactions were terminated by the addition of 5 mg/ml final concentration of heparin and loaded onto a native 5% polyacrylamide gel in 0.5 × TBE (90 mM Tris-borate and 2 mM EDTA, pH 8.3).

Northwestern analyses of brain or liver complexes were performed by exciting brain proteins bound to unlabeled NF-L RNA from native gels. A parallel identical binding reaction with radiolabeled NF-L RNA was used as an orientation marker for exclusion of relevant complexes. Proteins were eluted from acrylamide slices by incubation in protein elution buffer at 37°C for several hours and concentrated as described previously (Holcik and Liebhaber, 1997). Eluted proteins were fractionated in 10% SDS-PAGE, transferred to nitrocellulose, renatured, and hybridized with radiolabeled NF-L 68, 5K, plus 70 RNA, and poly(C). Hybridization and washing protocols were followed as described previously (Cañete-Soler et al., 2001).

RNA transcripts were synthesized by SP6 or T7 RNA polymerases (Promega) using appropriate templates in the presence of α32P-UTP or end-labeled with γ32P-ATP. Transcripts were un capped unless indicated otherwise. In that instance, the transcription reaction was performed, including 1 nM final concentration of synthetic cap structure (New England Biolabs, Beverly, MA). Radiolabeled and unlabeled transcripts used in competition assays were denatured at 90°C for 2 min and kept on ice for 1 min before addition to the binding reactions.

Western blot analyses were performed by fractionating 30–50 μg of cytoplasmic extracts or 5–50 ng of purified aldolases in 10% SDS-PAGE and transferred to nitrocellulose. For immunodetection, we used commercially available monoclonal HA and polyclonal aldolase A antibody (Santa Cruz Biotechnology) as well as monoclonal anti-Zebrin II (gift from Dr. Hawkes, University of Calgary, Calgary, Alberta, Canada). The ECL system (Amersham Biosciences) was used following the instructions of the manufacturer.

Circular dichroism. RNAs for circular dichroism (CD) were obtained by SP6/T7 transcription from appropriate templates, purified, and used in phosphate buffer, pH 7.4, at a final concentration of 1 μM. Purified aldolases, dialyzed against phosphate buffer, were used at a final concentration of 10 μM. Spectra were recorded on an AVIV 62DS spectropolarimeter (Aviv Associates, Lakewood, NJ) using rectangular quartz cells with a path length of 1 or 0.2 cm, respectively. Thermal control was maintained by a thermostatic unit with a Nestlab (Newington, NH) CFT-33 refrigerated, recirculating water bath and heat sink.

Cell transfection, immunoprecipitation, reverse transcription-PCR, and ribonuclease protection assays. Neuro-2a cells that express very low levels of NF-L and higher levels of the heavy neurofilament subunit (NF-H) were transfected with an HA-expressing vector or cotransfected with the neurofilament light subunit and respective HA aldolase A or aldolase C cDNAs. A luciferase reporter was included to correct for transfection efficiency. All transfections contained the same amount of DNA by adding vector DNA as needed. Cell lysates were harvested at 48 h after transfection and were HA-immunoprecipitated at a ratio of 9:1. Briefly, 900 μl of lysate was precipitated at 4°C for 2–4 h using a monoclonal HA-agarose conjugate antibody (Santa Cruz Biotechnology), and 100 μl was stored at 4°C as total cell lysate (control). The extent of immunoprecipitation was determined by Western blot analysis. All antibody-treated samples were spun, and the immunoprecipitates were washed and resuspended in elution buffer. All fractions, including one-tenth of the total cell lysate, were DNase I treated for 10 min at 37°C. RNA was obtained by phenol chloroform extraction and ethanol precipitation. RT was performed using the Superscript System (Invitrogen, San Diego, CA) and PCR amplification with specific primers from neurofilament transgene and vector sequences. To better assess the extent of product amplification, the PCR primers were supplemented with 0.1 μM end-labeled sense primer, and aliquots were taken after 12 and 22 cycles. The presence and integrity of mRNA, before and after immunoprecipitation, was assessed using specific primers for the endogenous neurofilament heavy subunit. To account for spurious amplification, we used specific primers for a 540 fragment of v-erb-B (Clontech).

Nonneuronal COS cells were obtained from the American Type Culture Collection and transiently transfected using Lipofectamine (3.5 μg/μg DNA; Invitrogen). Transfection mixtures contained the tetracycline transactivator (tTA) (pUHD15.1M) and NF-L wt, aldolase A, or
Results

Identification of neuronal aldolases A and C as neurofilament RNA-binding proteins

It was shown that a 68 nucleotide (nt) segment of the NF-L mRNA was essential for NF-L mRNA stabilization in neuronal cells (Cañete-Soler et al., 1998a) (Fig. 1A). Moreover, gel-shift assays revealed that one or more proteins present in neuronal tissue formed a stable complex with this RNA (Fig. 1B). The complex could be disrupted by adding an excess of poly(C). To identify NF-L mRNA binding proteins, we first performed Northwestern blotting (Wilusz, 1997) of a brainstem/spinal cord cDNA library for the following reasons: (1) the brain complex that forms on the NF-L 68 was very stable and therefore amenable to stringent washing protocols; (2) the experimental evidence that the 68 nt RNA probe was stable for ~1 h at 37°C; (3) the brain RNA-protein complex was sensitive to poly(C), and the property could be used to selectively identify NF-RNA binding factors that were not poly(C)-RNA binding proteins; and (4) the 68 nt RNA, used as a probe, could potentially identify NF-RNA transacting factors involved in regulating the stability of the transcript and/or involved in a motoneuron-like disease phenotype observed in transgenic mice. This library was selected because the brainstem and spinal cord express the highest levels of neurofilaments in the CNS. We first identified the neuronal exchange factor p190RhoGEF (Cañete-Soler et al., 2001). Three inserts of ~1.2 kb were partially sequenced (~600 bp), and the sequences matched those of aldolase (data not shown). Because the finding was unexpected, the interaction was probed by binding radiolabeled NF-L 68 nt RNA to proteins in brain extracts and examining the electrophoretic mobility shift (EMSA) of complexes formed under native conditions. With brain extract, an electrophoretic mobility shift was observed, whereas no shift was detected with liver extracts under identical conditions (Fig. 1B). SDS-PAGE and actin immunoblots showed that the protein content and integrity of the two extracts were similar (data not shown). A parallel EMSA with brain extracts and unlabeled NF-L 68 RNA was used to excise the complex from the native gel. The protein components involved in regulating the stability of the transcript and/or involved in a motoneuron-like disease phenotype were subsequently identified.

Figure 1. Detection of specific interactions between a 68 nt segment of neurofilament RNA and cytoplasmic extract from mouse tissues. A, Schematic diagram of NF-L mRNA showing location and sequence of destabilizing element. B, Electrophoretic mobility shift assay after incubation at 37°C of 1 ng of radiolabeled NF-L 68 with 50 μg of brain (lane 2) or liver (lane 3) extracts for 5 min before digestion with RNase T1. Binding reactions were analyzed on a native low-ionic strength 5% polyacrylamide gel. C, Identification of polypeptides in a brain extract RNA-protein complex by Northwestern analysis. A binding reaction identical to that shown in B (lane 2) was performed with unlabeled specific RNA probe and brain extract and resolved on a native 5% gel. The complex was excised from the gel, and the proteins were eluted and fractionated in a 10% SDS-PAGE. D, Western blot analysis of brain extract RNA protein complex. Proteins from brain RNA-protein complex in B (lane 2) were immunoblotted (IB) using anti-aldolase antibody. A control normal rabbit serum (NRS) is shown in lane 1, protein from brain ribonucleoprotein complex is shown in lane 2, and control purified aldolase is shown in lane 3.
To confirm that the 39 kDa protein was a major component of the RNA-protein complex, mouse brain cytoplasmic extracts were separated by 2D-gel electrophoresis (Fig. 2A), transferred to nitrocellulose, and hybridized separately to radiolabeled NF-L 68 or vector SK+/H100170. Two major spots of 39 kDa that specifically hybridized to the NF-L 68 RNA confirmed the EMSA studies. No specific binding was detected in filters hybridized with the SK+/H100170 nt RNA probe.

The identity of the 39 kDa proteins bound to NF-RNA was determined by Edman sequencing. The proteins were digested within gel slices with trypsin, and the peptides were extracted and separated by HPLC (Fig. 2B). Peptides corresponding to fractions 61 and 66 from the right spot (Fig. 2B, R) and fractions 36 and 42 from the left spot (Fig. 2B, L) were selected for Edman sequencing. The sequence from fraction 36 was unclear (XLQ(A/N)XALNA(W/T/S/Q/G) but corresponded to a tryptic peptide (ALQASALNAWR) from amino acids 304–314 (Fig. 2C).

The presence of N at position 8 (amino acid 311) in the sequence from fraction 36 distinguishes this as aldolase C (Pezza et al., 2003). The MW of this peptide determined by MALDI-MS was 1204.7 Da, which was close to the predicted mass of 1200.4 Da for this aldolase C peptide. Similarly, the sequence from fraction 42 was (A/G/S)VVPLAGT(G/D), which would correspond to the beginning of a tryptic peptide from amino acids 111–133, and the G or D at position 9 distinguishes this as aldolase A. Aldolase A has an N at this position (amino acid 119). One of the fragments from the right spot (fraction 66) corresponded to the most isozyme-specific tryptic fragment at the C terminus (Rottmann et al., 1987) (YTPSGQSGAASESFLISNHAY), which was clearly derived from aldolase A. The MALDI (Fig. 2C) confirmed that this was aldolase A with an observed mass of 2258.8 Da, which was approximately the same as the calculated mass of 2258.0 Da and 58 µm greater than that corresponding to aldolase C. The sequence from fraction 61 (GVVPLAGTNGWTTTQGLDG) was from the corresponding aldolase A peptide as that in fraction 42, but the sequence was much clearer. The location of identified sequences for aldolase A and C is shown in the supplemental material (available at www.jneurosci.org). These data confirmed that the brain extract 39 kDa proteins identified by one-dimensional (1D) Northwestern blotting (Fig. 1) were the neuronal glycolytic enzymes aldolase A and aldolase C.

The formation of a neurofilament RNA complex by brain extract is dependent on the presence of aldolase, restricted to the neuronal isozymes, and enhanced when the transcript is capped.

It is possible that aldolase, a major protein, is masking the actual binding protein in Northwestern analyses. To confirm the binding of aldolase to the NF-L 68 element, we performed EMSA using recombinant purified aldolase and brain extract as positive
As shown in Figure 3A, incubation of 0–80 μg of brain extract (lanes 1–4) or with 60–200 nM purified aldolase C (lanes 5–8). B, Formation of isozyme-dependent complexes after incubation of radiolabeled NF-L68 RNA with 10 nM purified aldolase A (lane 2), aldolase B (lane 3), or aldolase C (lane 4). C, Isozyme dose–response and magnesium requirements for complex formation. A total of 10–80 nM purified aldolase A (lanes 2–5), aldolase B (lanes 6–9), and aldolase C (lanes 10–13) was incubated with capped internally labeled NF-68 RNA, and complexes were resolved in a nondenaturing 5% acrylamide gel. Parallel binding reactions in the presence of 30 mM EDTA were conducted with 20–80 nM aldolase A (lanes 14–16) or aldolase C (lanes 17–19).

To determine whether the RNA-binding activity was isozyme dependent, all three aldolases (A, B, and C) were expressed in GST vectors, and the GST moiety was removed by PreScission protease. The GST-free aldolases were repurified using glutathione Sepharose 4B columns and dialyzed. Only aldolases A and C (Fig. 3B) were able to retard the migration of the riboprobe in a dose-dependent manner (Fig. 3A, lanes 5–8). A single complex in this case suggests that aldolase C has only one binding site in the uncapped transcript. Similar results were obtained with aldolase A (data not shown).

The affinity of aldolases for the neurofilament transcript was estimated by incubating increasing concentrations of respective purified aldolases (0–240 nm) with uncapped (Fig. 3B) or capped radiolabeled NF-L68 RNA (Fig. 3C). The $K_D$ of each aldolase was estimated from the plot of bound/free RNA versus aldolase concentration and was 30 ± 8 and 12 ± 8 nM for aldolase A and aldolase C, respectively. Binding of either neuronal aldolase to the transcript was dose dependent and saturable, reaching a plateau at 120–150 nM (data not shown).

The requirement for magnesium in the binding of aldolases to the transcript was investigated by performing the binding reactions with 30 mM EDTA or 5 mM magnesium. A capped radiolabeled NF-68 was allowed to bind aldolases at 37°C. In this instance, aldolase A formed one complex (Fig. 3C, lanes 2–5), whereas aldolase C formed two complexes (Fig. 3C, lanes 10–13). Aldolase A appeared to be magnesium independent, because the formation of the complex was not disrupted by the presence of EDTA (Fig. 3C, compare lanes 2–5 with lanes 14–16). In contrast, aldolase C binding reflected a dual behavior, because one of the complexes was disrupted with EDTA (Fig. 3C, top band; compare lanes 11–13 with lanes 17–19); however, the formation of the second complex was magnesium independent (Fig. 3C, top band; compare lanes 11–13 with lanes 17–19). Together, the findings show that the formation of a neurofilament RNA complex in brain extract is dependent on the presence of aldolase, is restricted to the two neuronal isozymes, and has high affinity. In addition, we show that the binding of aldolase C is enhanced when the transcript is provided with a cap structure and suggests that aldolase C could have different binding domains with differential preference for magnesium (Ross, 1999).

Specificity of aldolase binding to neurofilament transcripts

To assess the ability of aldolase to bind NF-L mRNA specifically and not RNA in general, other RNAs were used to compete with radiolabeled NF-L68 RNA (Fig. 4A). To this end, radiolabeled NF-L68 was incubated with aldolase C in the absence (Fig. 4A, lane 2) or presence of 10– to 100-fold excess of unrelated simian virus 40 RNAs (Fig.
4A, lanes 8–13) or 10- to 100-fold of two different adenosine–uracil (AU)-rich elements (lanes 14–19), a 35 nt AU-rich element of tumor necrosis factor α (TNFα) (Ford et al., 1999), and the AU-rich element of interleukin-3 (IL-3)/3’-UTR were unable to affect binding of aldolase to the NF-RNA, although a modest effect was observed with a 100-fold excess of TNFα and IL-3 adenosine-rich element (ARE). This indicated that aldolase binding to NF-L RNA is dependent on a specific sequence in the 68 nt RNA.

Direct and specific interaction of aldolase with the NF-L mRNA in solution was investigated by circular dichroism spectroscopy (Fig. 4B–D). Near-UV CD spectra from 240 to 320 nm for both the NF-L 68 and SK+70 RNAs were similar (Fig. 4B, C), with λ_{max} at 275 nm of 6 mdegrees (molar ellipticity). When aldolase A was added at 10-fold molar excess, however, only the spectra for the NF-L 68 RNA was affected (Fig. 4B), which indicated a possible change in RNA structure after binding. Far-UV CD was used to show that aldolases C and A were stabilized by interaction with NF-L 68 RNA (Fig. 4D). CD intensity was monitored as a function of temperature at 222 nm, a wavelength at which a spectral minimum exists for the α/β-barrel-fold of aldolase (Morris and Tolan, 1993). The thermal stability of aldolase A was greater than that of aldolase C with a T_{m} of 66 and 45°C, respectively. The addition of NF-L 68 RNA to aldolase C increased the thermal stability by 13°C, and the addition of this RNA to aldolase A increased its stability by 2°C. Data for both near-UV and far-UV CD support the formation of a complex between neuronal aldolases A and C and neurofilament mRNA.

Mapping of aldolase isozyme binding in the 680 nt mouse NF-L stability determinant region

The minimal sequence in the neurofilament mRNA required for binding of aldolases A and C was investigated by deletion analysis (Fig. 5). A 680 nt sequence of capped radiolabeled NF-L mRNA, including the complete 3’- UTR plus 250 nt of C-terminal coding region produced a mobility shift when incubated with aldolases A or C but not with aldolase B. Progressive deletion of 3’-UTR sequence did not decrease the binding affinity of aldolase A or C until 105 nt of 3’-UTR sequence was deleted. Binding of aldolase A or C was severely impaired after 320 nt was deleted with only 45 nt remaining. In contrast, both aldolases exhibited a shared focal site for binding. This focal point appeared to be the 68 nt element that includes 23 nt of distal coding region plus 45 nt of 3’-UTR and encompasses the translation termination site. In fact, binding of aldolase A to the 45 nt element was barely detectable but could be visualized if the transcript contained some 23 nt upstream of the stop codon. The findings are consistent with a previous observation (Cañete-Soler et al., 1998a) that the formation of a 3’-UTR neurofilament complex in brain extract was accentuated in the presence of distal coding region sequence. To ascertain that the binding of aldolase to the neurofilament transcript is species independent, we tested the ability of the isozymes to bind human NF-L 3’-UTR (Fig. 6). Aldolases A and C interacted with the human sequence (Fig. 6, lanes 2–4) as well as with the 45 nt of proximal 3’- UTR, harboring few base differences between the human and mouse neurofilaments (Fig. 6, lanes 9–16). The data are consistent with the notion that binding of aldolase to NF-L mRNA is species independent.

Aldolases A and C interact with NF-L mRNA in vivo

The interaction between the neuronal aldolases and the light neurofilament mRNA occurs in vivo. It was assessed by immunoprecipitation of HA-tagged aldolase A or aldolase C expressed in Neuro-2a cells transfected with a transcribable full-length NF-L cDNA. Neuro-2a cells express very low levels of light NF-L but express the heavy NF-H subunit at levels that are detectable by ribonuclease protection assay. Aldolase expression is also low. Cells were cotransfected with both constructs and immunoprecipitated using a monoclonal HA antibody. Immunoprecipitation efficiency was confirmed by Western blot analysis (Fig. 7A). The nucleic acid was extracted from the immunocomplexes, and RT-PCR was performed using specific primers from the HA vector and neurofilament transgene sequence. The neurofilament transgene primers were selected from sequences 45 nt apart. Primers for the endogenous NF-H, which gives a 617 PCR product, were used as a control for the integrity of RNA in all samples as well as to test for specificity of the mRNA-protein interaction. To better assess the extent of product amplification, the PCRs...
To confirm the in vivo significance of aldolase binding to the neurofilament (NF-L) mRNA, Neuro-2a cells were transfected with HA aldolase C or cotransfected with a tetracycline-inducible NF-L and HA aldolase C. Transfections included tTA and the luciferase reporter to account for transfection efficiency (Fig. 8). mRNA rates of degradation were assessed by ribonuclease protection assay. Parallel experiments were performed to compare the decay of endogenous neurofilaments (L, M, and H) with that of the light (NF-L) transgene. We used mouse β-actin as a loading control. Cells transfected with HA aldolase alone revealed detectable but very low levels of endogenous NF-L as well as medium-sized NF (NF-M) and NF-H. However, whereas the NF-L mRNA degraded with time, the levels of NF-M and NF-H were unchanged (Fig. 8, lanes 1–3 and 7–9). Cells cotransfected with aldolase C and NF-L exhibited high levels of NF-L after induction (Fig. 8, lane 4) but declined quickly after the addition of 0.5 μg/ml doxycycline to deactivate transcription (Fig. 8, lanes 5, 6), indicating that aldolase C increased the decay rates of the neurofilament transgene. In contrast, the presence of aldolase did not affect the half-life of either endogenous M or H (Fig. 8, compare lanes 4–6 with lanes 10–12). The findings support a preferential binding and activity of aldolase on the NF-L mRNA.

To assess the effect of expression of aldolases A and C on the decay of NF-L mRNA in a heterologous system, COS cells were transfected with inducible NF-L and HA aldolase C or cotransfected with a tetracycline-inducible NF-L and HA aldolase C or cotransfected with a tetracycline-inducible NF-L and HA aldolase C. Transfections included tTA and the luciferase reporter to account for transfection efficiency (Fig. 8). Rates of NF-L mRNA degradation were evaluated as above. Cotransfection of NF-L wt and aldolases A and C (Fig. 9A, lanes 1–4) exhibited NF-L mRNA degradation rates similar to those reported previously (Canete-Soler et al., 1998b). Cells cotransfected with mutant NF-L lacking 250 nt of distal coding region (Fig. 9A, lanes 5–8) or with deleted 3´-UTR (Fig. 9A, lanes 9–12) showed decreased rates of degradation. The lower signal shown by β-actin reflects a greater susceptibility of COS cell endogenous β-actin to RNase treatment in protection assays because of sequence differences. The findings are consistent with a major role of aldolase in modulating the half-life of the light neurofilament in vivo, because deletion of sequences necessary for binding of aldolase stabilizes the transcript. Moreover, they confirm a correlation between in vitro binding to NF-L and a functional effect.

Expression of aldolases and the light neurofilament in nonneuronal cells excludes the endogenous PABP from the complex

The NF-L mRNA is highly conserved among species and is expressed in nonneuronal cells such as neuroblastoma cells. Therefore, we assessed the effect of expression of aldolases A and C on the decay of NF-L mRNA in Neuro-2a cells. The findings are consistent with a major role of aldolase in modulating the half-life of the light neurofilament in vivo, because deletion of sequences necessary for binding of aldolase stabilizes the transcript. Moreover, they confirm a correlation between in vitro binding to NF-L and a functional effect.

Coexpression of aldolases and the light neurofilament in nonneuronal cells excludes the endogenous PABP from the complex

To address potential mechanisms underlying the regulatory effect of aldolase in NF-L mRNA decay, we tested whether aldolases interacted with the PABP within the NF-RNA protein complex. To this end, COS cells were cotransfected with NF-L and HA-tagged aldolases (Fig. 10). Ribonucleoprotein (RNP) complexes

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**Figure 5.** Mapping of protein-binding in the 680 nt mouse neurofilament light stability determinant region. The 680 nt encompasses the full 3´-UTR plus 250 nt of distal coding region of the neurofilament light subunit sequence. Binding reactions were conducted as described in Figure 1. Electrophoretic mobility shift assays of radiolabeled 0.68 kb RNA sequence (lane 1) or shorter variants depicted in the top panel (lanes 5, 9, 13, 17, 21, 25) were conducted to test for their ability to form band-shifted complexes. The full-length construct was used to assess the contribution of the 3´-UTR (lane 1) and 5´-UTR (lane 2) sequences. The findings confirmed that the interaction between neurofilament mRNA and aldolases A and C occurs in vivo, and the interaction is specific for the NF-L mRNA.

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**Table 1. Isozyme Binding**

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Ectopic expression of aldolase increases the degradation rate of the NF-L mRNA
were immunoprecipitated with monoclonal PABP or HA antibodies and immunoblotted with polyclonal PABP or aldolase C antibodies. Ribonucleoprotein complexes enriched in PABP by immunoprecipitation revealed the presence of the protein in the RNP lysate and immunopellet, and aldolase was found only in the lysate (Fig. 10A). Conversely, when the ribonucleoprotein complexes were immunoprecipitated with HA antibodies, PABP was found only in the lysate but not in the immunopellet (Fig. 10B). The findings indicate a competitive interaction between aldolase and PABP within the NF-mRNA protein complex and suggest that the modulation of neurofilament stabilization is effected, at least by a competing interaction between aldolases and PABP.

Aldolases A and C exhibit a cap-like-associated ribonucleolytic activity in vitro The enhancement of aldolase C binding to a capped NF-L 68 transcript was striking and led us to ask whether the aldolases might have a cap-associated nucleolytic activity. To determine whether this was the case, we incubated a cap-labeled NF-L 68 with 100 nM purified aldolase C at 37°C for 60 min. Aliquots were taken at varying times and electrophoresed (Fig. 11B). Aldolase C was able to generate intermediates that retained radioactivity at their 5'-ends. In contrast, when aldolase was incubated with uncapped internally labeled NF-68, no degradation occurred (data not shown). We then sought to restore the cap-like-associated activity by complementation with a factor(s) present in brain extracts (Fig. 11B). Uncapped end-labeled NF-68 was incubated with aldolase in the absence (Fig. 11C, lane 2) or presence of increasing concentrations of brain extract (Fig. 11C, lanes 3–8). Without brain extract, aldolase C was unable to degrade uncapped end-labeled transcript (Fig. 11C, compare input lane 2 with lane 3). However, addition of 1.6 μg of brain extract was sufficient to initiate a decay process that generated intermediates (Fig. 11C, lanes 4–7). Incubation of the uncapped end-labeled NF-L68 with brain extract in the absence of aldolase C recapitulated the decay process, indicating that an additional cap-independent degradation activity is present in brain extract (Fig. 11D, lanes 1–4). The fragmentation pattern and the size of intermediates suggest that aldolase is an endonuclease, likely the same or very related endonuclease found to be associated with polyribosomes (Lee et al., 1998) and shown to interact with the poly(C) -binding protein (Wang and Kiledjian, 2000); however, the possibility...
ity of a distributive exonuclease cannot be discounted (Ross, 1999). A careful examination of the neurofilament sequence revealed the existence of two AUUAG motifs, one of them some 300 nt upstream of the stop codon and the second one at the translation termination site. The findings suggest that aldolase C could be the same endoribonucle- 
ase that cleaves β-globin mRNA at UG sites and could be part of a specialized decay pathway that overlaps the general non-sense- 
mediated decay pathway (Stevens et al., 2002). Moreover, a previous report on hu-
man β-globin mRNAs harboring a nonsense codon has shown that the degradative inter-
mediates bound an anti-cap antibody and were resistant to 5’-to-3’ exonuclease activity (Lim and Maquat, 1992). Together, the find-
ings suggest that the neuronal aldolases are nuclease that cleave the neurofilament tran-
script at specific UG sites. Their activity might be dependent on a yet unidentified 
cellular activity that “marks” the transcript at its 5’-end to be recognized by aldolases A 
and C. Validation of this possible mecha-
nism awaits additional studies.

Discussion
This report identifies the neuronal aldola-
oses A and C as components of an NF-L 
mRNA complex and describes novel, spe-
cific interactions between the glycolytic 
enzymes and the neuronal transcript. These interactions are not fortuitous in 
that we show that the enzymes regulate the 
stability of NF-L mRNA in vivo. We also 
begin to address the mechanisms whereby 
alдолases A and C partake in regulating 
NF-L mRNA stability by demonstrating 
that the enzymes exhibit ribonuclease ac-
tivity and undergo competitive interac-
tions with the PABP in vivo. Our findings 
indicate that aldolases A and C are instru-
mental components of a ribonucleoprotein 
complex that modulates the stability of the NF-L transcript, possibly by activa-
tion of their ribonucleolytic activity. It is 
unclear whether the role of aldolases in 
mediating high-energy consumption in 
large neurons may be related to their reg-
ulation of NF-L mRNA expression.

Aldolases are glycolytic enzymes cata-
yzing reactions in the glycolytic, glu-
coneogenic, and fructose metabolic path-
ways. Three vertebrate isozymes (A, B, and 
C) are conserved through evolution (Be-
rardini et al., 1997). They are tetrameric 
enzymes and do not possess classical RNA-
binding motifs (Burd and Dreyfuss, 1994). 
Aldolase A is expressed predominantly in 
muscle and brain, aldolase B is expressed 
predominantly in liver, and aldolase C is 
expressed predominantly in brain (Leb-
Aldolase C may also have moonlighting activities in establishing subsets of neurons during cerebellar development (Hawkes et al., 1993; Ahn et al., 1994) or during differentiation of progenitor cells in the subventricular zones of the developing brain (Staugaitis et al., 2001). Like neurofilament transcripts, aldolases A and C are expressed early in embryonic development, are upregulated during postnatal life (Kusakabe et al., 1997; Shiokawa et al., 2002), and are also differentially expressed in complementary cell types (Walther et al., 1998). Aldolase binds to the actin cytoskeleton and mediates the association of F-actin with the insulin-responsive glucose transporter GLUT4 (Kusakabe et al., 1997; Wang et al., 1997; Kao et al., 1999; Jewet and Sibley, 2003); however, the association with a neuronal mRNA was only recently uncovered (Cañete-Soler et al., 2003).

The interaction between aldolase and NF-L mRNA is restricted to the two neuronal isoforms. Why the mammalian brain needs two very similar glycolytic enzymes is not understood (Rottmann et al., 1987). The differential pattern of expression of aldolases A and C in brain is reminiscent of the shifting pattern of Hu expression during neuronal differentiation in mouse (Okano and Darnell, 1997), chicken (Wakamatsu and Weston, 1997), Xenopus (Perron et al., 1999), and zebrafish (Park et al., 2000). It suggests that sets of RNA-binding proteins in varying composition in ribonucleoprotein complexes (Keene and Tenembaum, 2002) may have differing effects on differentiation in subsets of neurons. It has been shown that the neuronal ELAV (embryonic lethal abnormal vision)-like protein (Hel-N1) does not affect the steady-state levels of NF-M mRNA, despite the fact that Hel-N1 binds to the 3′-UTR of NF-M and associates directly with the transcript in transfected cells. However, it was observed that cells overexpressing Hel-N1 had NF-M actively translated, because it migrated with the heavy polysome fraction and suggested that expression of Hel-N1 favored reinitiation of NF-M translation (Antic et al., 1999). Like neurofilament transcripts, aldolases A and C are expressed early in embryonic development, are upregulated during postnatal life (Kusakabe et al., 1997; Shiokawa et al., 2002), and are also differentially expressed in complementary cell types (Walther et al., 1998). Aldolase binds to the actin cytoskeleton and mediates the association of F-actin with the insulin-responsive glucose transporter GLUT4 (Kusakabe et al., 1997; Wang et al., 1997; Kao et al., 1999; Jewet and Sibley, 2003); however, the association with a neuronal mRNA was only recently uncovered (Cañete-Soler et al., 2003).

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It is also conceivable that differential composition of RNP complexes in subsets of neurons could determine their differential susceptibility to degenerative conditions or vulnerability to adverse mutations. We have shown previously that a mutant NF-L transgene, with a disrupted configuration at the junction of the coding region and 3′-UTR (Cañete-Soler et al., 1999), or overexpression of neurofilament RNA sequences placed in the 3′-UTR of a GFP reporter gene (Nie et al., 2002) has profound neuropathic effects on motor neurons in mice. Here, we show that coexpression of NF-L mRNA and aldolase C leads to an increased rate of degradation of the NF-L transcript. Moreover, the degradation rate is inhibited when aldolases are coexpressed with mutant NF-L that lacks mRNA sequence for binding of aldolase. The findings raise the possibility that disruption of a homeostatic neurofilament NF-L mRNA complex might occur by the following: (1) titrating out a component(s) of the complex if the NF-L mRNA is misexpressed (Xu et al., 1993; Bergeron et al., 1994; Nie et al., 2002), or (2) preventing adequate binding of
alpha lactalbumin if the proper contiguity between coding region and 3′-UTR is altered (Lee et al., 1994; Cañete-Soler et al., 1999; Amrani et al., 2004). In either case, alterations in the number of NFs might not be the cause of the disease phenotype but rather the effect of the aberrant positioning or composition of a complex that becomes the target of disruptive interactions among specific and general components of the postsynaptic machinery.

Alldoses A and C bind with different affinities to the NF-L, and their differential affinity is also manifested in differential ribonuclease activity. This raises the intriguing possibility that cells that coexpress alldoses A and C have heterometramers that bind and act differently than the homometramers present in cells that express one isoform or the other. That the NF-L 68 element binds so effectively to alldose contrasts with the poor binding of NF-L 680ΔΔ, despite the fact that NF-L 680ΔΔ contains also the 23 nt upstream of the stop codon. It suggests that there must be a negative element (anti-determinant) upstream in the coding region, which, when removed, allows full binding of alldose. It is also possible that the function of the 68 nt element in vivo is to approximate sequences in the coding region with those in the 3′-UTR. This structural proximity could be necessary for interaction between the two isozymes and specific activation of their activity in subsets of neurons. A similar strategy has been reported to occur in tissue-specific regulation of alternative splicing (Baraniak et al., 2003). There is also increasing evidence that RNA-association factors or impair recycling of translational components or impair recycling of translational components (Decker and Parker, 1995; Mangus et al., 2003; Amrani et al., 2004). This, in turn, might have effects in trans with deleterious consequences for the normal expression of other gene products (Lim et al., 2004; Ge et al., 2005). The latter becomes critical for large neurons with extraordinary high metabolic demands at specific sites (Wu et al., 1997).

In summary, this study provides novel insights in the field of RNA-protein interactions in the nervous system. It shows that proteins believed to have metabolic functions also regulate the expression of a prominent neuron-specific mRNA. Moreover, it underscores the complexities of the gene expression circuitry for regulating neuronal function and dysfunction (Keene, 2003).

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