p190RhoGEF Binds to a Destabilizing Element in the 3' Untranslated Region of Light Neurofilament Subunit mRNA and Alters the Stability of the Transcript*

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Stabilization of neurofilament (NF) mRNAs plays a major role in regulating levels of NF expression and in establishing axonal size and rate of axonal conduction. Previous studies have identified a 68-nucleotide destabilizing element at the junction of the coding region and 3' untranslated region of the light NF subunit (NF-L) mRNA. The present study has used the destabilizing element (probe A) to screen a rat brain cDNA library for interactive proteins. A cDNA clone encoding 1068 nucleotides in the C-terminal domain of p190RhoGEF (clone 39) was found to bind strongly and specifically to the RNA probe. The interaction was confirmed using a glutathione S-transferase/clone 39 fusion protein in Northw estern, gel-shift, and cross-linkage studies. The glutathione S-transferase/clone 39 fusion protein also enhanced the cross-linkage of a major 43-kDa protein in brain extract to the destabilizing element. Functional studies on stably transfected neuronal cells showed that p190RhoGEF expression increased the half-life of a wild-type NF-L mRNA but did not alter the half-life of a mutant NF-L mRNA lacking the destabilizing element. The findings reveal a novel interactive feature of p190RhoGEF that links the exchange factor with NF mRNA stability and regulation of the axonal cytoskeleton.

NF expression is regulated by transcriptional and post-transcriptional mechanisms. Whereas the restriction of NF expression to neurons is determined at the level of transcription, the amounts of NF gene products expressed in different neurons are regulated post-transcriptionally (6). Although there is also evidence of translation control (7, 8), a major component of post-transcriptional regulation occurs at the level of the mRNA stability (9). The latter phenomenon is exemplified by the simultaneous increase in NF subunit expression during postnatal development (2), mediated by stabilization of their respective transcripts (10). Stabilization of NF mRNAs remains an active process in differentiated neurons as evidenced by the sudden and coordinated destabilization of NF transcripts following nerve transection (10). Thus, mRNA stability serves as a means for regulating NF expression and maintaining levels of NF expression commensurate with the size and extent of the axonal conduit.

Insights into the localization of cis-acting elements regulating the stabilities of NF transcripts were obtained from patterns of transgene expression in mice bearing wild-type and mutant NF light subunit (NF-L) transgenes (6). These studies showed that the postnatal up-regulation and axotomy-induced down-regulation of NF-L mRNA are regulated by elements in the 3' UTR of the NF-L transcript. Functional studies of mRNA stability then identified a 68-nucleotide (nt) element at the junction of the coding region and 3' UTR whose deletion leads to a marked stabilization of the NF-L transcript (11). The 68-nt destabilizing element (probe A) is highly interactive with proteins in brain extract and neuronal cell lines using gel-shift and cross-linking methods (12). However, there is still no information as to the identity of these trans-acting factors or their effects on NF-L mRNA stability.

The present study has begun to identify the regulatory factors that bind to the destabilizing element and to assess their potential role in regulating NF-L mRNA stability. For this purpose, the 68-nt destabilizing element (probe A) was used as an RNA probe to screen for interactive proteins in a rat brain cDNA library (see Ref. 13). To our surprise, we identified the C-terminal domain of p190RhoGEF as a binding partner to the destabilizing element. We then confirmed the interactions by biochemical methods and showed that p190RhoGEF alters the stability of NF-L mRNA in vivo. The identification of p190RhoGEF in post-transcriptional control of NF-L expression provides a starting point for probing specific interactions among RNA-protein components on the NF-L transcript and their possible connections to other regulatory pathways in the neuron.

EXPERIMENTAL PROCEDURES
cDNA Cloning—A rat brain λ ZapII cDNA library (Stratagene) was screened with an RNA probe to sequence between cDNA 1712 and 1779 of mouse NF-L mRNA (11). This sequence was transcribed in SK+ vector
by T7 polymerase to generate probe A (10⁶ cpm/ml). Approximately 2 × 10⁶ plaques were screened with the radioactive probe (5 × 10⁶ cpm/ml). Four plasmids with 1.2-kb inserts were isolated by in vivo excision, and their cDNAs were sequenced in both directions using the dideoxynucleotide chain termination method. The largest cDNA (clone 39) was subcloned into the EcoRI sites of pGEX-6P (Amersham Pharmacia Biotech). GST protein and GST-clone 39 fusion protein were expressed in BL21 bacteria and purified according to standard protocols (Amersham Pharmacia Biotech). A full-length p190RhoGEF cDNA was provided by Dr. Wouter Moolenaar (14). Clone 39 and p190RhoGEF cDNAs were inserted in-frame into pEGFP-C1 or pEGFP-C2 expression vectors provided by Dr. Wouter Moolenaar (14). Clone 39 and p190RhoGEF cDNAs were sequenced in both directions using the dideoxynucleotide chain termination method. The largest cDNA (clone 39) was subcloned into the EcoRI sites of pGEX-6P (Amersham Pharmacia Biotech). GST protein and GST-clone 39 fusion protein were expressed in BL21 bacteria and purified according to standard protocols (Amersham Pharmacia Biotech). A full-length p190RhoGEF cDNA was provided by Dr. Wouter Moolenaar (14). Clone 39 and p190RhoGEF cDNAs were inserted in-frame into pEGFP-C1 or pEGFP-C2 expression vectors (CLONTECH), and pEGFP-C1, pEGFP/clone 39, and pEGFP/p190RhoGEF expression vectors were transfected into Neuro2a cells.

Northern Blot—20 μg of total rat brain RNA were hybridized with cDNA probes to a Kpn/EcoRI fragment from the 5′ end of p190RhoGEF (14) and to a BamHI/XhoI fragment from clone 39. Radioactive probes were prepared using 3²P-dCTP (10⁶ cpm/μg) and a random prime labeling kit (Roche Molecular Biochemicals).

Northwestern and Western Blots—Purified GST and GST-clone 39 fusion proteins (5 μg), lysates (50 μg) of Neuro2a cell transfected with pEGFP-C1, pEGFP/clone 39, or pEGFP/p190RhoGEF, and brain extract (100 μg) were separated on SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, and reacted with probe A (10⁶ cpm/ml) as described (15). Parallel samples of transfected Neuro2a cell lysates were also transferred to Immobilon polyvinylidene difluoride membranes (Roche Molecular Biochemicals) and immunoblotted with anti-green fluorescent protein Living Colors antibody (CLONTECH), and immunoreactivity was detected using a chemiluminescence kit (Roche Molecular Biochemicals). Expression of EGFP, EGFP/p190RhoGEF, and EGFP/clone 39 fluorescence was observed in vivo and estimated to occur in 20–40% of cells. Brain extracts were obtained by centrifugation (100,000 × g for 1 h) of rat brain homogenates (12).

Assessment of NF-L mRNA Stability—NF-L mRNA stability was assessed using Neuro2a cells containing the tTA transactivator cDNA and either wild-type (NF-L+/++) or mutant (L+/del/+) NF-L cDNA under the control of a Tn-10 tetracycline-inducible promoter (11). These cells were stably transfected with a full-length p190RhoGEF cDNA in a pcDNA3.1 vector or with the vector alone, selected with hygromycin, and pooled (100 clones). The presence and expression of p190RhoGEF cDNA in the modified cells were confirmed by polymerase chain reaction and protection assay.

Cells were grown in the absence of tetracycline, split into replicate plates, and harvested in triplicate for assessment of NF-L mRNA at varying time points after readdition of tetracycline. Levels of mRNA from wild-type and mutant NF-L transgenes were normalized to levels of β-actin mRNA. mRNAs were assessed by ribonuclease protection assay.
C-terminal Domain of p190RhoGEF Binds NF mRNA

RESULTS

C-terminal Domain of p190RhoGEF (Clone 39) Binds an RNA Probe to the Destabilizing Element of the NF-L mRNA—To identify RNA-binding proteins that participate in regulating the stability of NF-L mRNA, a rat brain cDNA library was probed with the 68-nt sequence to the stability determinant of NF-L mRNA (probe A). Four clones encoding the same cDNA insert were identified. The largest (clone 39) encoded a 1068-nt open reading frame that is 92% identical to the C terminus of mouse p190RhoGEF (14) (Fig. 1, A and B). Although the sequence does not contain known RNA binding motifs, there are several regions enriched in arginine residues with predicted α-helical structures that may have RNA binding activity (16–20).

To determine whether clone 39 might be a truncated isoform of p190RhoGEF, Northern blots of rat brain mRNA were probed with cDNA probes to the 5’ and 3’ ends of p190RhoGEF. Both probes hybridized to 5.5- and 7.0-kb mRNAs (Fig. 1C), similar to the sizes of p190RhoGEF transcripts described previously in mouse brain (14).

To confirm the RNA binding properties of the C-terminal domain of p190RhoGEF, purified extracts of bacteria expressing a GST-clone 39 fusion protein were reacted with probe A by Northwestern blot (Fig. 1D). Binding to a 68-kDa protein occurred in extracts containing the fusion protein but not in extracts containing the GST vector alone or in extracts from uninduced bacteria. The 68-kDa protein is the expected size of a GST-clone 39 fusion protein. p190RhoGEF C-terminal domain (clone 39) enhances the binding of 43-kDa protein in brain extracts to the NF-L mRNA stability determinant.

The RNA binding properties of the GST-clone 39 fusion protein were further tested by gel retardation assay and by cross-linkage of RNA-protein complexes (Fig. 2). Additions of fusion protein (5–50 nM) led to a dose-dependent formation of a retarded radioactive band when binding reactions were electrophoresed on non-denaturing gels (Fig. 2A). The formation of the retarded band did not occur using a nonspecific RNA probe or upon addition of 500 nM of unfused GST protein. Radioactivity from probe A was cross-linked to a 68-kDa GST-clone 39 fusion protein (Fig. 2B). Cross-linkage was competed with unlabeled probe A but not by a nonspecific RNA probe.

Cross-linkage of probe A to the 68-kDa GST-clone 39 fusion protein was then compared with cross-linkage of probe A to a 43-kDa protein in brain extracts (Fig. 2C). The 43-kDa protein is the major RNA-binding protein in the poly(C)-sensitive complexes that bind to and regulate the stability of the NF-L (12) and NF-H (15) mRNAs. Cross-linkage to the 43-kDa protein was competed with poly(C) but not by poly(U), whereas cross-linkage to the 68-kDa GST-clone 39 fusion protein was competed by both homoribopolymers. Whereas cross-linkage to the 68-kDa protein decreased when the GST-clone 39 fusion protein was supplemented with brain extract, cross-linkage to the 43-kDa protein was markedly increased in the presence of GST-clone 39 fusion protein. Addition of fusion protein caused at least a 10-fold increase in binding of 43-kDa protein to low concentrations of brain extract (Fig. 2D).

The lack of cross-linkage of probe A to endogenous p190RhoGEF in brain extract could be because of the large size of the native protein or because of the relatively small amounts of the protein in high speed supernatants of brain extract. Binding of probe A to clone 39 and full-length p190RhoGEF was therefore tested using lysates of Neuro2a cells expressing clone 39 or p190RhoGEF as EGFP fusion proteins. Parallel samples of lysates were separated by SDS electrophoresis, transferred to membranes, and either immunoblotted with an EGFP antibody (Fig. 2E) or reacted with probe A (Fig. 2F). Both clone 39 and full-length p190RhoGEF fusion proteins, but not the unfused EGFP protein, bound to the radioactive probe.

Together, the findings demonstrate binding of the C-terminal domain, as well as the full-length p190RhoGEF, to RNA sequence in the mRNA stability determinant of NF-L. Moreover, binding to the C-terminal domain alters the binding of a 43-kDa RNA-binding protein in brain extracts to the same
C-terminal Domain of p190RhoGEF Binds NF mRNA

sequence. Because the latter protein is implicated in regulating NF mRNA stability (11, 12, 15), the C-terminal domain of p190RhoGEF may effect or be affected by alterations in NF mRNA stability. Expression of p190RhoGEF alters the stability of a wild-type NF-L mRNA but not the stability of a mutant NF-L mRNA lacking the destabilizing element.

A functional role of p190RhoGEF in altering NF-L mRNA stability was tested by examining the effects of p190RhoGEF expression on levels of wild-type NF-L mRNA or levels of mutant NF-L mRNA lacking the destabilizing element in transcript (11). Wild-type and mutant NF-L cDNAs were placed as transgenes in a tetracycline-inducible system so that high levels of transgenic mRNAs could be induced, and rates of mRNA decay could be assessed following addition of the tetracycline ligand to turn off further transcription of the transgenes. p190RhoGEF expression was brought about by stably transfected a pcDNA expression vector containing the full-length p190RhoGEF cDNA.

High levels of wild-type NF-L mRNA (NF-L in Fig. 3A) or an NF-L mRNA lacking a destabilizing element (NF-L(del) in Fig. 3B) were obtained by withdrawal of tetracycline from the medium. Rates of mRNA decay were determined by assessing NF mRNA levels at 0 (lanes 1 and 5), 24 (lanes 2 and 6), 48 (lanes 3 and 7), and 72 (lanes 4 and 8) h after addition of tetracycline to inactivate the transgene. Lane 9 shows the lack of endogenous NF-L gene expression in parental Neuro2a cells. B, protective assays of an NF-L mRNA lacking destabilizing element (NF-L(del)) and β-actin mRNA in Neuro2a cells containing p190RhoGEF expression vector (+RhoGEF) or vector alone (−RhoGEF) and tetracycline-inducible system with mutant NF-L cDNA target. The experimental protocol was identical to that used for A. C, quantitation of NF-L/β-actin mRNA levels in Neuro2a cells containing wild-type NF-L transgene, as described for A. D, quantitation of NF-L(del)/β-actin mRNA levels in Neuro2a cells containing mutant NF-L transgene lacking instability determinant, as described for B. A and B show representative experiments, and C and D were averaged from four experiments.

Four separate experiments is presented in Fig. 3, C and D. p190RhoGEF expression stabilized the wild-type NF-L transcript (Fig. 3C) but not the mutant NF-L mRNA lacking a destabilizing element (Fig. 3D). Hence, deletion of the stabilizing element increases mRNA stability but renders the transcript insensitive to the effects of p190RhoGEF expression. A nearly 2-fold increase of NF-L mRNA stability was achieved either by deleting the destabilizing element or by expression of p190RhoGEF.

Similar results were observed in an initial series of studies using transient transfections of target NF-L constructs and EGFP marker protein fused to the C-terminal domain of p190RhoGEF (clone 39) or to the full-length p190RhoGEF protein (data not shown). Variability in the extent of transient transfections and their expressions over time precluded an accurate assessment of change.

**DISCUSSION**

This study has identified and characterized interactive properties of p190RhoGEF that implicate the exchange factor in post-transcriptional regulation of neuronal gene products. The C-terminal domain of the exchange factor binds to an RNA sequence in the 3′ UTR of NF-L transcript and links a component of the Rho kinase pathway with the RNA-protein complex regulating NF-L mRNA stability. Binding of the C-terminal domain of p190RhoGEF (clone 39) to the NF-L stability determinant (probe A), initially detected in a cDNA library screen, was confirmed by Northwestern blot, gel-shift, and cross-linkage studies. The latter findings solidify the RNA binding fea-
tuties of the exchange factor, because Northwestern, gel-shift, and cross-linkage studies measure different, albeit complementary, aspects of RNA-protein interactions.

Neither the interactions among components of RNA-protein complexes nor their roles in regulating NF-L mRNA stability are presently understood. It is, therefore, unclear to what extent the p190RhoGEF-dependent alterations in NF-L mRNA stability may relate to the ability of the C-terminal domain of p190RhoGEF to enhance the binding of a 43-kDa protein in brain extracts to the NF-L mRNA stability determinant. The 43-kDa protein is the principal cross-linked component of RNA-protein complexes that assemble on destabilizing elements in the 3′ UTRs of the NF-L and NF-H mRNAs (15). It is possible that allosteric alterations from protein-protein or protein-RNA interactions could facilitate binding of the 43-kDa protein to the destabilizing element. On the other hand, the role of the 43-kDa protein in regulating RNA stability is completely unknown. Recent studies have just begun to unravel the complex mechanisms whereby RNA-protein complexes increase or decrease the stabilities of specific mRNAs (21–23).

The introduction of a tetracycline-inducible system to test RNA stability enables transcription of specific target genes to be manipulated and avoids the pleiotropic effects from using transcription inhibitors (11). This system was used to identify the 68-nt destabilizing element at the junction of the coding region and 3′ UTR of NF-L (11). The system was herein applied to examine the effects of p190RhoGEF expression on the stability of the NF-L transcript. The results showed that expression of p190RhoGEF alters the stability of the NF-L transcript and that alteration of NF-L mRNA stability was dependent on the presence of the destabilizing element in target transcript. The findings also demonstrate that the interactive properties of p190RhoGEF could be conveyed in the context of a full-length p190RhoGEF protein.

The ability to bind and alter the stability of NF-L mRNA represents a novel and unexpected feature of p190RhoGEF. p190RhoGEF was initially identified in a two-hybrid screen as a brain-enriched, Rho-specific GDP/GTP exchange factor with the ability to mimic Rho-like cytoskeletal changes when transfected in a neuronal cell line (14). We confirmed the presence of 4.0- and 7.2-kb p190RhoGEF mRNAs species in rat brain, as initially reported in mouse brain (14). We also showed that each mRNA hybridized to cDNA probes to the 5′ and 3′ ends of the p190RhoGEF coding region, indicating the N- and C termini of p190RhoGEF are not excluded from either isoform. Although the nature and derivation of the different p190RhoGEF isoforms remain unclear, it is unlikely that the RNA binding properties of the C-terminal domain have evolved in a separate isoform. Furthermore, the functional effects of p190RhoGEF as an exchange factor and of altering NF-L mRNA stability were demonstrated using the same p190RhoGEF cDNA (14). More recently, p190RhoGEF has been shown to require an unknown binding partner to unmask its GDP/GTP exchange activity in vitro (24). Binding of p190RhoGEF to the NF-L mRNA stability may be a component of this activation.

It is noteworthy that the prospective roles of p190RhoGEF in activation of Rho GTPase and in post-transcriptional modulation of NF-L expression would involve the functioning of p190RhoGEF during differing time frames of neuronal development. Although Rho GTPases are highly instrumental in mediating cytoskeletal changes during early neuronal differentiation (25), their role in differentiated neurons is less clear. On the other hand, post-transcriptional regulation of NF-L expres-

sion becomes operational during late neuronal development and remains active in the fully differentiated state. Hence, the preferential expression in differentiated neurons (26), and especially the prominent expression of p190RhoGEF mRNA in fully mature murine brain (14), is consistent with a role of p190RhoGEF in the post-transcriptional regulation of NF-L expression. Moreover, in situ hybridization reveals high levels of p190RhoGEF mRNA in large motor neurons commensurate with the high levels of NF metabolism in these cells.2

Interactions of p190RhoGEF with the destabilizing element in the NF-L mRNA could serve to modulate mRNA stability or, alternatively, could reflect changes in stabilities of the transcripts. In either case, the potential role of p190RhoGEF in neuronal homeostasis is intriguing in view of the strategic role that stabilization of NF transcripts serves in maintaining corresponding levels of NF expression and axonal size of individual neurons. Indeed, a possible involvement of p190RhoGEF in regulating the homeostasis of motor neurons derives from the unexpected neuropathic effects brought about by mutating the destabilizing element in an NF-L transgene (27). The latter finding implicates the interactions of trans-acting factors with the destabilizing element in mediating the neuropathic effects of the transgene. The identification of p190RhoGEF as a possible modulator of motor neuron homeostasis warrants further study.

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