Binding of p190RhoGEF to a Destabilizing Element on the Light Neurofilament mRNA Is Competed by BC1 RNA*

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The enhancement of RNA-mediated motor neuron degeneration in transgenic mice by mutating a major mRNA instability determinant in a light neurofilament (NF-L) transgene implicates cognate RNA binding factors in the pathogenesis of motor neuron degeneration. p190RhoGEF is a neuron-enriched guanine exchange factor (GEF) that binds to the NF-L-destabilizing element, to c-Jun N-terminal kinase-interactive protein-1 (JIP-1), and to 14-3-3 and may link neurofilament expression to pathways affecting neuronal homeostasis. This study was undertaken to identify additional RNA species that bind p190RhoGEF and could affect interactions of the exchange factor with NF-L transcripts. The C-terminal domain of p190RhoGEF, containing the RNA-binding site, was expressed as a glutathione S-transferase fusion protein and was used as an affinity probe to isolate interactive RNAs in rat brain extracts. As expected, NF-L mRNA was identified as an RNA species eluted from the affinity column. In addition, BC1 RNA was also found enriched in the bound RNA fraction. BC1 is a 152-nucleotide RNA that is highly expressed but untranslated in differentiated neurons. We show that BC1 and NF-L mRNA bind to a similar site in the C-terminal domain of p190RhoGEF, and their bindings to p190RhoGEF are readily cross-competed. Moreover, we identify a novel binding site in BC1 to account for its interaction with p190RhoGEF. The findings suggest a novel role of BC1 in differentiated neurons involving RNA-protein interactions of p190RhoGEF.

Motor neuron degeneration occurs in transgenic mice expressing high levels of a neurofilament light subunit (NF-L) transgene (1) or neurofilament heavy subunit transgene (2) but not in mice expressing high levels of a neurofilament mid-sized subunit transgene (3). A more severe form of motor neuron degeneration occurs in mice expressing low levels of an NF-L transgene with a leucine-to-proline mutation in the rod domain and a c-Myc tag appended to the C-terminal end of the protein (4). A severe form of motor neuron degeneration also occurs from low level expression of an NF-L transgene lacking the point mutation but with the c-Myc tag at the end of the coding region (5). The latter finding raised the possibility that neuropathic effects result from expression of a mutant mRNA by the transgene. This interpretation is based on the location of the c-Myc tag in a major destabilizing element in NF-L mRNA and the ability of the c-Myc insert to stabilize the transcript as well as alter the binding of cognate binding factors in brain extracts to the NF-L mRNA destabilizing element by gel-shift and cross-linking assays (5). More recently, motor neuron degeneration was reproduced in transgenic mice when the destabilizing element of NF-L mRNA was placed in the 3′-untranslated region of an enhanced green fluorescent protein reporter transgene (6).

p190RhoGEF was identified as a potential component in RNA-mediated motor neuron degeneration by virtue of its ability to bind to the stability determinant and alter NF-L mRNA stability (7) as well as its interaction with JIP-1 (8), microtubules (9), and 14-3-3 (10). More recently, we have shown that overexpression of p190RhoGEF protects Neuro-2a cells from stress-induced apoptosis, possibly by interacting in the JIP-1/ c-Jun N-terminal kinase pathway. Moreover, p190RhoGEF is a neuronal enriched exchange factor that is up-regulated during postnatal development and highly expressed in large differentiated neurons, including motor neurons in mouse spinal cord. A similar expression profile is exhibited by NFs (11) and by neuron-enriched isoforms of JIP-1 (12) and 14-3-3 (13–15). The involvement of the NF-L-destabilizing element in RNA-mediated motor neuron degeneration raised the possibility that additional RNA-protein interactions of the C-terminal domain of p190RhoGEF may be instrumental in modulating RNA-mediated neuropathic effects. To pursue this line of study, the C-terminal domain of p190RhoGEF (p190RhoGEF-C) containing the RNA-binding site was fused to GST protein and used as affinity matrix for identifying additional interactive RNA species in soluble brain extracts. To our surprise, we identified BC1 as an RNA with high affinity for the RNA-binding site in p190RhoGEF. BC1 is an untranslated 152-nucleotide polymerase III transcript that is up-regulated during postnatal development (16) and highly expressed in large neurons of rat brain and spinal cord (17, 18). The importance of BC1 in neuronal function is not yet fully defined, but the favored view is that BC1 serves as a molecular scaffold for regulating transport and translation of neuronal mRNAs at dendritic sites (19, 20). The binding of BC1 and NF-L mRNA to the same binding site in p190RhoGEF, as reported herein, suggests that BC1 may play a role in RNA-protein interactions of p190RhoGEF and in modulating the effects of the exchange factor in large differentiated neurons.

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1 The abbreviations used are: NF-L, neurofilament light subunit; GEF, guanine exchange factor; JIP-1, c-Jun N-terminal kinase-interactive protein-1; GST, glutathione S-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; aa, amino acids; HA, hemagglutinin; RT, reverse transcriptase; DTM, dendrite-targeting motif.

2 R. Cañete-Soler, unpublished observations.

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EXPERIMENTAL PROCEDURES

DNA Constructs—A cDNA-encoding sequence between amino acids (aa) 1276–1582 in the C-terminal domain of p190RhoGEF was fused in-frame to glutathione-S-transferase (GST) in pGEX-6P (Amersham Life Science). Membranes were preincubated with Blotto A (10× Tris-HCl, pH 8.0, 150 mM NaCl, 5% milk) on shaker at 4 °C overnight and incubated with 5 × 10^4 cpm/ml probe in hybridization buffer for 1 h at room temperature. Membranes were extensively washed with hybridization buffer, and dried membranes were subjected to overnight exposure for autoradiography.

RESULTS

BC1 and NF68 Bind to and Cross-Compete for p190RhoGEF—The C terminus of p190RhoGEF (p190 RhoGEF-C) was isolated from a rat brain cDNA library by virtue of its binding to a 68-nucleotide RNA probe (NF68) comprising a destabilizing element in mouse NF-L mRNA (7). To identify additional RNA targets of p190RhoGEF-C, the cDNA was fused to GST, and fusion protein was used as an affinity matrix to screen a high speed supernatant fraction of rat brain. Bound RNAs were recovered from the beads, reverse-transcribed into cDNAs, and the cDNAs cloned and sequenced. The resulting cDNAs not only included NF-L but also revealed more frequent recovery of short elements matching the sequence of BC1. The structure and partial sequence of BC1 is shown in Fig. 1.

To verify the binding of BC1 to p190RhoGEF-C, GST/p190RhoGEF-C (150 pmol) was spotted on nylon membrane, along with equimolar amounts of unfused GST protein (GST) and bovine serum albumin (BSA), and reacted with uniformly labeled full-length BC1 probe (Fig. 2). BC1 bound to p190RhoGEF-C but not to BSA or to unfused GST protein. A similar-sized RNA probe to sequence adjoining the T7-binding site on BC1 was situated within the ID region of BC1 RNA (see below).

Gel retardation assays were undertaken to assess the abilities of BC1 and NF68 probes to alter the electrophoretic migration of GST/p190RhoGEF. Fig. 3 shows that incubation of GST/p190RhoGEF-C with 32P-labeled NF68 or BC1 probes generates similar radioactive bands (arrow). The radioactive gel-retarded band formed with NF68 (3rd lane) was competed by 100-fold excesses of unlabeled NF68 (4th lane) or unlabeled BC1 (5th lane) but not by an unlabeled nonspecific SK+ probe (data not shown). Likewise, the gel-retarded band formed with BC1 (6th lane) was competed by 100-fold excesses of unlabeled NF68 (7th lane) or unlabeled BC1 (8th lane) but not by an unlabeled SK+ probe (data not shown). Incubation of GST/p190RhoGEF with 32P-labeled SK+ probe did not generate a gel-retarded band (9th lane).

In Vivo Binding of BC1 RNA to p190RhoGEF Protein—Lysates from Neuro-2a cells transfected with HA-tagged p190RhoGEF-C protein or pHM6 vector alone were immuno-

**Fig. 1. Schematic diagram of BC1.** The 152-nucleotide (nt) RNA is composed of ID, A-rich, and unique sequences. Nucleotide composition of the proximal (A), middle (B), and distal (C) segments of the ID sequence is shown. The initial 10 nucleotides of the ID sequence consist of a dendritic targeting motif (DTM).
precipitated with agarose-conjugated anti-HA monoclonal antibody. The immunoprecipitates were washed extensively and solubilized by boiling in 0.1% SDS. RT-PCR assays were conducted on the solubilized fractions to assess the presence of BC1 RNA in the respective immunoprecipitates. Fig. 4 shows that BC1 RNA (arrow) was recovered from Neuro-2a cells transfected with HA-tagged p190RhoGEF-C (lane 1) but not from Neuro-2a cells transfected with the empty expression vector (lane 2). The size of the BC1 RT-PCR fragment was 175 bp, including T7 promoter sequence in upstream primer, and is shown with a 100-bp marker standard (lane M).

Binding Site on BC1 RNA for GST/p190RhoGEF-C—Full-length and truncated GST/p190RhoGEF-C were constructed for localizing the NF68-binding site by Northwestern blots. The same full-length and truncated GST fusion proteins were used to localize and compare the BC1- and NF68-binding sites. GST fusion proteins were expressed in bacteria and purified by glutathione affinity chromatography. Purified proteins were electrophoresed in SDS-PAGE (Fig. 5A) and transferred to nylon membranes, and replicates of transferred proteins were hybridized with BC1 (Fig. 5C) and NF68 (Fig. 5D) probes by Northwestern blots. Full-length and N-terminal truncations (M4 and M5 constructs) of GST/p190RhoGEF-C bound 32P-labeled BC1 (Fig. 5C) and NF68 (Fig. 5D) probes. Binding of both BC1 and NF68 probes was lost in C-terminal truncations of GST/p190RhoGEF-C. Whereas binding was retained when the C terminus of GST/p190RhoGEF-C was truncated from aa 1582 to 1524 (M3 construct), binding of NF68 and BC1 probes was eliminated upon C-terminal truncations to aa 1493 (M2 construct) and 1461 (M1 construct). The findings indicate that BC1 and NF68 bind to similar sites in GST/p190RhoGEF and that sequence encoded between aa 1493 and 1524 (QLQEYQQLRLEQGKMERQKMKVRQGL) is required for the binding of both RNA probes.

Binding Site on BC1 RNA for GST/p190RhoGEF-C—GST/p190RhoGEF-C was separated by SDS-PAGE and transferred to nylon membrane for Northwestern blots to determine the sequence in BC1 required for binding to the fusion protein. Membrane containing fusion protein was hybridized with full-length BC1 and with a series of truncated probes lacking 5’ or 3’ sequences (Fig. 6A). 32P-Labeled probes to full-length BC1 (BC1), to ID sequence (ID), and to the full-length BC1 probe lacking the initial 10 nucleotides of the dendrite-targeting motif or DTM (BC1ΔDTM) bound to the fusion protein (Fig. 6B). Binding of 32P-labeled probes to the fusion protein was lost when the 5’ deletion was extended to include the initial 25 (BC1ΔID-A) or 50 (BC1ΔID-A/B) nucleotides of BC1. The findings indicated that sequence within the ID element of BC1 was sufficient for binding and that sequences in the initial 25 nucleotides of BC1, excluding the DTM, are necessary for binding of BC1 to GST/p190RhoGEF-C.

3 J. Wu, unpublished data.
DISCUSSION

Novel binding features of BC1 are herein identified which expand the prospective role of BC1 in neuronal metabolism. Binding of BC1 and the destabilizing element in NF-L mRNA to a similar site in the C-terminal domain of p190RhoGEF links BC1 to NF-L expression and to RNA-mediated motor neuron degeneration (7). The recognition of p190RhoGEF as an RNA-binding protein also links NF-L expression with signal transduction pathways and with potential modifications of the neuronal cytoskeleton in differentiated neurons (21–23). Moreover, the identification of a novel binding site outside of the dendrite-targeting motif (DTM) supports the likelihood that BC1 has additional functional properties beyond its putative role in regulating movement, localization, and translation of neuronal transcripts in the dendritic compartment (19, 20).

There is presently much more information as to the derivation than function of BC1. For example, BC1 is recognized as a master gene for generating and amplifying short interspersed repetitive elements in the rodent genome (24–26) analogous to the Alu repeats in the primate genome (27–29). BC1 and other progenitor genes are believed to have arisen by reverse transcription and by fortuitous integration of cDNAs into a favorable site of the genome downstream of a pol III promoter sequence. BC1 is, however, an atypical pol III transcript in that it is highly and exclusively expressed in differentiated neurons (30). Neuron-specific transcription is regulated by positive and negative upstream elements that react with brain-specific and nonspecific factors (18). Competitive binding of Pur protein to the BC1 transcript and to cis-acting enhancer elements in the BC1 gene provides a potential feedback mechanism for regulating BC1 transcription by its own gene product (31).

The up-regulation of BC1 during the late phase of neuronal differentiation and high level expression in large differentiated neurons (16) coincides with the expression profiles of NF-L (11) and p190RhoGEF and is consistent with a functional interaction of these components in large differentiated neurons. A similar expression profile is also characteristic of interactive partners of p190RhoGEF, including neuron-enriched isoforms of JIP-1 (12) and 14-3-3 (13–15). It is possible that binding of one or more of the interactive partners to the C-terminal domain could be responsible for unmasking inherent GDP/GTP activity required for activation of p190RhoGEF in vivo (9). It is also possible for interaction of BC1 with p190RhoGEF to account for axonal transport or localization of BC1 in discrete sites along the surface membrane of goldfish Mauthner axons (32). It should be noted, however, there are still large regions in the N-terminal half of p190RhoGEF containing potential inter-
motor neuron degeneration could provide some insights into the nature of motor neuron disease.

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