Nerve growth factor controls GAP-43 mRNA stability via the phosphoprotein ARPP-19

Nina Irwin*†‡, Steven Chao*§, Luda Goritchenko*, Atsuko Horiuchi¶, Paul Greengard¶, Angus C. Nairn¶‖, and Larry I. Benowitz*†‡§

*Department of Neurosurgery, Children's Hospital, Boston, MA 02115; †Department of Surgery and §Program in Neuroscience, Harvard Medical School, Boston, MA 02115; ¶Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021; and ‖Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06508

Contributed by Paul Greengard, July 31, 2002

The membrane phosphoprotein GAP-43 is involved in axon growth and synaptic plasticity. In PC12 pheochromocytoma cells, induction of a neuronal phenotype by nerve growth factor (NGF) is accompanied by a marked increase in GAP-43 levels. NGF regulates GAP-43 expression by altering the half-life of its mRNA. We report here that the phosphoprotein ARPP-19 mediates this regulation. In an NGF-dependent manner, ARPP-19 bound to a region in the 3′ end of GAP-43 mRNA previously found to be important for regulating the half-life of the mRNA. Overexpression of wild-type ARPP-19 in PC12 cells increased the NGF-dependent expression of a reporter construct linked to the critical 3′ region of GAP-43 mRNA. Mutation of serine 104, the site of phosphorylation by protein kinase A in ARPP-19, to either alanine or aspartate abolished this regulation in PC12 cells. These findings demonstrate that ARPP-19 is an important link between NGF signaling and post-transcriptional control of neuronal gene expression.

The growth-associated protein-43 (GAP-43) is involved in the development and plasticity of the nervous system. Elevated expression of GAP-43 promotes neurite outgrowth *in vivo* and in cultured cells. Conversely, suppression of GAP-43 expression disrupts growth cone formation and axon pathfinding (1–6). In addition, elevated expression of GAP-43, together with a related protein CAP-23, leads to axonal sprouting in spinal cord (7). GAP-43 can associate with PIP(4,5)P_2-rich domains in the nerve terminal membrane, where it regulates actin polymerization (8, 9). During the course of neuronal development or regeneration, the expression of GAP-43 varies over a 100-fold range, from very low levels in resting neurons to high levels in cells undergoing axogenesis or synaptic remodeling (10, 11). Although GAP-43 expression declines precipitously in most neurons after mature synapses have formed, it persists at high levels in associative areas of the brain, where the protein may contribute to changes in synaptic function (12) and to structural remodeling (6, 11, 13). Thus, the mechanisms that control GAP-43 expression are important for understanding the regulation of neuronal outgrowth and plasticity.

In PC12 pheochromocytoma cells, nerve growth factor (NGF) stimulates GAP-43 expression by altering the stability of its mRNA (14–16). NGF treatment of PC12 cells increases the half-life of GAP-43 mRNA from 5–6 h to 30 h (14, 15), which allows the mRNA to accumulate and be translated into protein. NGF-mediated regulation of GAP-43 mRNA stability has been linked to particular domains in the 3′ end of the mRNA spanning the end of the coding region and the beginning of the 3′ UTR (17). NGF regulates the stability of chimeric mRNAs containing this region but fails to do so if this region is deleted from the reporter construct (17). In addition, expression of high levels of the 3′ UTR of GAP-43 mRNA blocks NGF induction of endogenous GAP-43, presumably by titrating proteins that otherwise bind to and stabilize the mRNA (18).

In this study, we have identified a protein that mediates the regulation of GAP-43 mRNA stability by NGF. Although prior studies have identified proteins that bind to the 3′ end of GAP-43 mRNA (19, 20), none of these proteins binds to the specific region that is crucial for mediating NGF-dependent stabilization, nor does NGF regulate the binding of these other proteins to GAP-43 mRNA. Here, we report that cAMP-regulated phosphoprotein-19 (ARPP-19) binds to the region in the 3′ end of GAP-43 mRNA implicated in regulating its half-life in an NGF-dependent manner. Elevated expression of ARPP-19 in PC12 cells enables reporter constructs linked to the critical 3′ region of GAP-43 mRNA to be regulated by NGF. Moreover, expression of an ARPP-19 mutant, in which the phosphorylated serine was replaced by alanine or aspartate, reduced both basal and NGF-dependent regulation. These findings demonstrate a link between NGF signaling and stabilization of GAP-43 mRNA that may be involved in neuronal differentiation.

Methods

RNA Synthesis. Plasmid pF1-1, provided by courtesy of A. Rosenthal (Genentech) and A. Routtenberg (Northwestern University, Evanston, IL), contains a 1.5-kb insert (see Fig. 1) encoding the 5′ UTR, coding sequence, and most of the 3′ UTR of GAP-43 mRNA cloned into the *Eco*RI site of pGEM3 (Promega). The *Pst*I-*Eco*RI fragment encodes the 3′ half of the mRNA, and digestion of this with *Sau*IIIA produces four small fragments (Fig. 1*a*) that we cloned into pGEM3. Region A, which encodes RNA-binding region 2 (*rbr2*), contains 180 bases between the *Pst*I and first *Sau*IIIA sites and includes the last 38 codons and the first 66 untranslated nucleotides. Region B is 114 nt in length, region C is 116 nt, and region D, encoding *rbr1*, extends from the third *Sau*IIIA site to the *Eco*RI site at the end of the clone. Each plasmid was linearized with *Eco*RI and transcribed *in vitro* from the SP6 promoter in the presence of [α-^{32}P]UTP (DuPont/NEN).

Northwestern and Immunoblot Assays. Northwestern assays were performed as described (21). Cytoplasmic proteins or purified, recombinant proteins were separated by SDS/PAGE and transferred to nitrocellulose (Bio-Rad). After blocking with 5× Denhardt's solution in TES (10 mM Tris·Cl, pH 7.4/0.5 mM EDTA/50 mM NaCl) containing 0.1 mM DTT for 2 h, 10 ng/ml ^{32}P-labeled RNA was added and the incubation was continued for 1 h. The filter then was washed once with TES plus 1× Denhardt's solution and 0.1 mM DTT and twice with TES plus 0.1 mM DTT, each for 10 min at room temperature. The filter was exposed to Kodak XAR film overnight at 4°C.

For immunoblot analyses, proteins were separated by SDS/PAGE and transferred to poly(vinylidene difluoride) membranes (Millipore). Membranes were incubated overnight at 4°C with a rabbit anti-ARPP-19 antibody (22) in Tris-buffered saline

Abbreviations: TBS, Tris-buffered saline; CMV, cytomegalovirus; ARPP-19, cAMP-regulated phosphoprotein-19; EGFP, enhanced GFP; GAP-43, growth-associated protein-43; NGF, nerve growth factor; rbr, RNA-binding region; PKA, protein kinase A.

‡To whom reprint requests may be addressed. E-mail: nina.irwin@tch.harvard.edu or larry.benowitz@tch.harvard.edu.

NEUROSCIENCE

(TBS) containing 5% milk and 2% goat serum. After three washes in TBS containing 0.5% Triton X-100 and 2× NaCl (TBS_2T), membranes were incubated for 2 h at room temperature in 1:1,000 dilution of an HRP-conjugated goat anti-rabbit antibody in TBS_2T containing 5% milk and 2% normal serum. Membranes were washed twice in TBS_2T and once in TBS. Immunoblots were developed by using the ECL (enhanced chemiluminescence) detection system (Amersham Pharmacia).

Gel-Shift Assays. Binding reactions used standard methods (23). Each 10-μl reaction volume contained 4 μl of a protein sample, 5 μl of 2× binding buffer [30 mM Hepes, pH 7.4/20 mM KCl/20% glycerol/10 mM $MgCl_2$/0.4 mM DTT/0.2 mg/ml heparin/1.2 units/μl RNasin (Promega)], and 1 μl (approximately 10 ng) of ^{32}P-labeled RNA. After incubation at room temperature for 15 min, RNase A1 (90 units) was added and the incubation was continued for an additional 10 min. Radiolabeled RNA fragments and protein–RNA complexes were separated on nondenaturing, RNase-free 0.25× TBE, 4% polyacrylamide gels prerun at 25 V at 4°C for 30 min. After loading the binding reactions onto the running gel, separation continued at 15 $V \cdot cm^{-1}$ for 1.5 h at 4°C. Protein–RNA complexes were visualized by autoradiography with Kodak XAR film (4°C overnight).

Protein Purification. PC12 cells were homogenized in buffer containing 20 mM Tris·Cl, pH 7.4/50 mM KCl/5 mM EGTA/5 mM $MgCl_2$/0.1 mM DTT/1 μg/ml leupeptin/0.1 mM PMSF and centrifuged at 12,000 × *g* for 15 min. Trichloroacetic acid was added to the supernatant to a concentration of 2%. After a 15-min incubation, acid-insoluble proteins were removed by centrifugation at 12,000 × *g* for 15 min. The supernatant was transferred to a fresh tube, and trichloroacetic acid was added to a final concentration of 20% to precipitate the remaining soluble proteins. The precipitate was neutralized with 1.5 M Tris·Cl, pH 8.8, the proteins were separated on a 15% SDS/PAGE gel, and binding activity was assayed by Northwestern blotting (21). The isolated protein was digested with trypsin, and peptides were separated by reversed-phase HPLC. The Harvard Microchemistry Facility carried out amino acid sequencing. Recombinant rat ARPP-19 was expressed and purified as described (24).

Reporter Assays. PC12 cells were cotransfected with an expression and a reporter plasmid. The former was a plasmid encoding wild-type or mutant rat ARPP-19 with a cytomegalovirus (CMV) promoter or a control vector plasmid (pcDNA3; Invitrogen). The latter was a reporter encoding enhanced GFP (EGFP) with a CMV promoter (pEGFP-C, Invitrogen), linked to one of the four 3′ fragments of GAP-43 mRNA shown in Fig. 1. Twenty-four hours after calcium phosphate-mediated transfection, cells were glycerol-shocked for 3 min and incubated in the absence or presence of NGF (50 ng/ml; Sigma) for 3 days. Cells were collected and lysed by freeze-thaw. Fluorescence was measured by using a fluorometer (Turner, Palo Alto, CA); protein concentrations were determined by Bradford assays (Bio-Rad). In other experiments, fluorescent cells were counted in each well of a 24-well culture plate. Every experiment included four wells for each condition, randomly distributed on a 24-well tissue culture plate and counted blind. To make ARPP-19 mutants, the serine 104 codon was converted through site-directed mutagenesis to either an alanine (A) or aspartate (D) codon. The *rbr2*Δ642–764 mutant was created by PCR mutagenesis.

Results

To identify trans-acting factors that might mediate the effects of NGF on GAP-43 mRNA stability, we examined the binding of radiolabeled GAP-43 mRNA fragments to cytosolic proteins of PC12 cells. Initial experiments used a *Pst*I-*Eco*RI fragment representing the end of the coding region and most of the 3′ UTR (Fig. 1*a*). Treating cells with NGF resulted in a time-dependent increase in the binding of a 19-kDa protein to this mRNA fragment (Fig. 2 *Upper*). A control probe showed little binding activity (Fig. 2 *Lower*).

To further define the binding region, we subcloned four nonoverlapping fragments within the 3′ end of GAP-43 mRNA (Fig. 1*a*). The only region capable of binding to the 19-kDa protein was *rbr2* (Fig. 1*b*). The *rbr2* fragment includes the end of the coding region and the first 66 nt of the 3′ UTR and shows a high degree of evolutionary conservation (Fig. 1*c*). A gel-shift assay was used to further characterize the interaction of *rbr2* with the 19-kDa protein. The specificity of the binding was demonstrated by its displacement with a 100-fold molar excess of

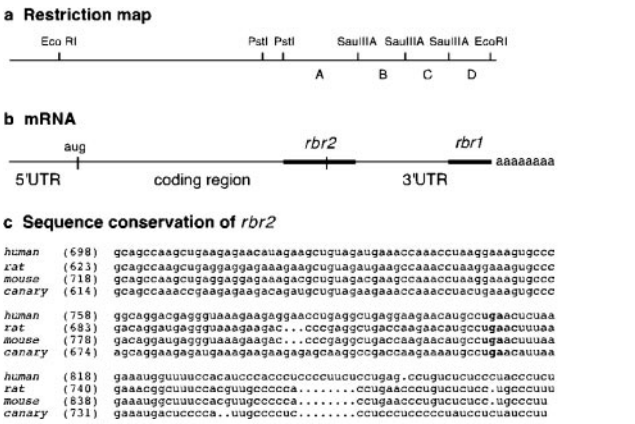

Fig. 1. Structure of rat GAP-43 mRNA. (*a*) Restriction map of rat GAP-43 cDNA showing *Eco*RI, *Pst*I, and *Sau*IIIA sites. Fragment A extends from the *Pst*I site to the first *Sau*IIIA site and encodes *rbr2*. Fragments B and C are the *Sau*IIIA fragments. Fragment D extends from the last *Sau*IIIA site to the *Eco*RI site and encodes *rbr1*. (*b*) Most GAP-43 mRNA transcripts contain a 52-nt 5′ UTR, a coding region of 630 nt, and a 3′ UTR of about 600 nt. (*c*) *rbr2* shows a high degree of conservation among human, rat, mouse, and canary.

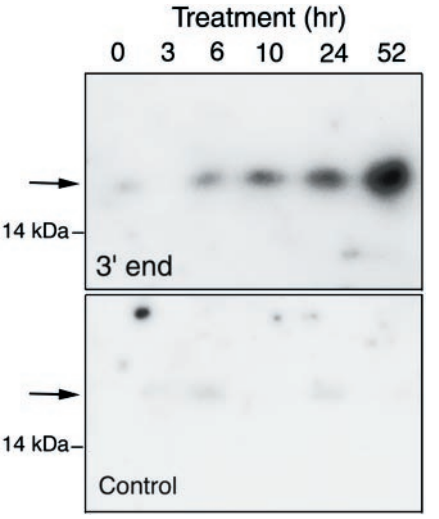

Fig. 2. NGF regulates the binding of a 19-kDa protein to the *rbr2* region of GAP-43 mRNA. PC12 cells were treated for the indicated times with NGF. Proteins were separated by SDS/PAGE, transferred to nitrocellulose, and incubated with a radiolabeled GAP-43 RNA probe transcribed from the 3′ *Pst*I-*Eco*RI fragment shown in Fig. 1*a*. The arrow points to a 19-kDa protein whose binding to the 3′ region of GAP-43 mRNA increases with time of NGF treatment. A control probe derived from bacteriophage lambda sequences is shown below (Control).

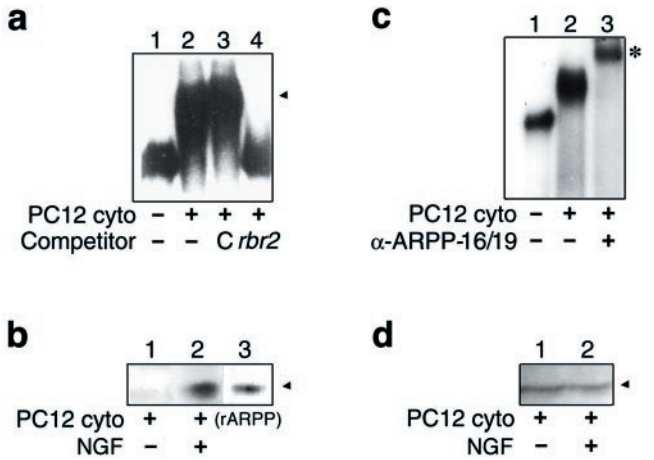

Fig. 3. Analysis of ARPP-19 binding to the *rbr2* region. (*a*) Gel-shift assays were used to evaluate the specificity of the binding reaction. Lane 1, migration of radiolabeled *rbr2* alone; lane 2, cytoplasmic proteins from NGF-treated PC12 cells retard the migration of *rbr2* (arrowhead); radiolabeled *rbr2* is not displaced from the complex by a 100-fold molar excess of 3′ UTR fragment C (lane 3: see Fig. 1), but is displaced by a 100-fold molar excess of unlabeled *rbr2* RNA (lane 4). (*b*) NGF alters ARPP-19 binding to *rbr2*. Proteins from untreated (lane 1) and NGF-treated (lane 2) PC12 cells, along with recombinant ARPP-19 (lane 3), were separated by SDS/PAGE, transferred to nitrocellulose, and probed with radiolabeled *rbr2*. NGF causes increased binding of a 19-kDa protein to *rbr2 (*lane 2 vs. 1), and this protein comigrates with recombinant ARPP-19, which also shows binding activity. (c) Supershift assays. Lanes: 1, radiolabeled *rbr2* alone; 2, migration of *rbr2* shifted by cytoplasmic proteins from NGF-treated PC12 cells; 3, ''supershift'' of the protein–RNA complex by the addition of an anti-ARPP-16/19 antibody (*). (*d*) NGF does not alter ARPP-19 expression levels. Proteins from untreated or NGF-treated PC12 cells were separated by SDS/PAGE, transferred to nitrocellulose, and probed with a rabbit anti-ARPP-16/19 antibody. ARPP-19 levels are similar in untreated (lane 1) and NGF-treated (lane 2) PC12 cells.

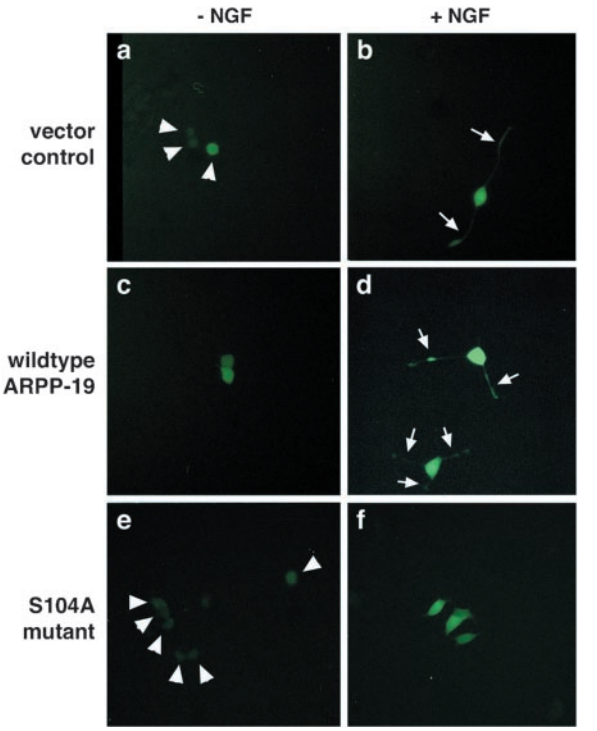

Fig. 4. NGF plus ARPP-19 stimulate expression of an EGFP-*rbr2* reporter. PC12 cells were cotransfected with a reporter plasmid expressing EGFP from a CMV promoter with *rbr2* inserted downstream of EGFP and a control vector plasmid, pcDNA3 (Invitrogen) (*a* and *b*), or a plasmid expressing wild-type ARPP-19 (*c* and *d*) or the Ser104Ala (S104A) mutant of ARPP-19 (*e* and *f*), all from a CMV promoter. After transfection, cells were grown in the absence (*a*, *c*, and *e*) or presence (*b*, *d*, and *f*) of NGF. Arrows point to neurites that are induced by NGF treatment. Arrowheads point to faint cells expressing low levels of the reporter EGFP. The cells in *a–e* were selected to illustrate the intensity of cell staining, whereas the cells in *f* are more intensely stained than average but were selected to illustrate the absence of processes (compare with *d*).

nonradioactive *rbr2* but not by excess RNA from another part of the 3′ UTR (fragment C, Fig. 3*a*). *rbr2* largely coincides with the region previously shown to be crucial for mediating the effect of NGF on GAP-43 mRNA stability (17).

We purified the 19-kDa mRNA-binding protein from the cytosol of NGF-treated PC12 cells by using differential acid solubility and gel electrophoresis. After tryptic digestion and sequencing, a partial amino acid sequence, YFDSGDYNMAK, was identified that matched precisely a segment of the proteins ARPP-16 and ARPP-19 (25). These proteins initially were identified in brain as substrates for cAMP-dependent protein kinase A (PKA) with apparent molecular masses of 16 and 19 kDa on SDS/PAGE (25). The proteins are produced by alternative splicing of a single gene and differ only by the addition of 16 aa at the N terminus of ARPP-19. Both ARPP-16 (96 aa) and ARPP-19 (112 aa) exhibit anomalous migration on SDS/PAGE.

Recombinant ARPP-19 was found to comigrate on SDS/PAGE with the *rbr2*-binding protein of 19 kDa from PC12 cells (Fig. 3*b*). Furthermore, recombinant ARPP-19 bound directly to radiolabeled *rbr2* (Fig. 3*b*). To further characterize the interaction of ARPP-19 and *rbr2*, we examined the ability of an antibody to ARPP-16/19 to modify the migration of the complex in the gel-shift assay. The addition of the anti-ARPP-16/19 antibody ''supershifted'' the electrophoretic migration of the protein–RNA complex formed when PC12 cell extracts were mixed with *rbr2* (Fig. 3*c*).

To directly examine whether binding of ARPP-19 to *rbr2* regulates gene expression, we used reporter constructs containing the EGFP gene linked to one of the four regions in the 3′ end of the GAP-43 gene shown in Fig. 1*a*. PC12 cells were cotransfected with the reporter plasmid plus a plasmid expressing either (*i*) wild-type ARPP-19, (*ii*) mutant ARPP-19, or (*iii*) the vector pcDNA3 as a control. Cells were grown for 3 days in the absence or presence of NGF. Expression of the EGFP-*rbr2* reporter increased 2- to 3-fold above baseline when cells were transfected with the ARPP-19 expression plasmid and treated with NGF (Figs. 4 *a–d* and 5 *a* and *c*). In contrast, reporter constructs containing any of the other three regions of GAP-43 mRNA 3′ UTR failed to show increased expression in response to NGF and elevated ARPP-19 (Fig. 5 *a* and *c*). A deletion mutation that removed the central third of the *rbr2* fragment eliminated the response of the reporter to NGF and elevated ARPP-19 (Fig. 5*d*). These results strongly implicate *rbr2* as the cis regulatory region and ARPP-19 as the transactivator of GAP-43 mRNA stability.

NGF treatment did not alter the overall level of ARPP-19 in PC12 cells (Fig. 3*d*), raising the possibility that the effect of ARPP-19 on GAP-43 mRNA stability may involve a posttranslational modification. ARPP-19 is phosphorylated at Ser-104 by PKA (24). To test whether Ser-104 might play a role in the regulation of GAP-43 mRNA stability, two ARPP-19 mutants, Ser-104→Ala and Ser-104→Asp, were coexpressed with the *rbr2* reporter construct in PC12 cells. The Ser-104→Ala mutant reduced expression of the EGFP reporter to levels below that seen when cells were transfected with the pcDNA3 vector alone (Figs. 4 *a* and *e* and 5*b*) and attenuated NGF-mediated neurite outgrowth (Fig. 4 *f* vs. *b*). Similar results were seen with the Ser-104→Asp mutant (Fig. 5*b* and data not shown). However,

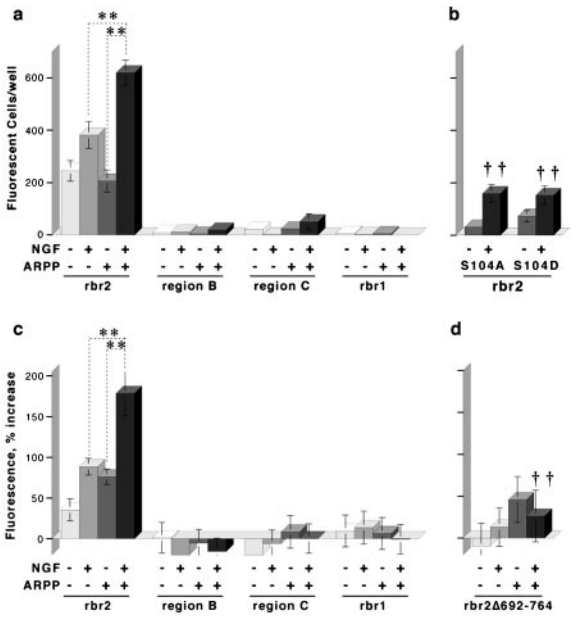

Fig. 5. Regulation of mRNA stability: quantitative studies. PC12 cells were cotransfected with a reporter plasmid expressing EGFP from a CMV promoter linked to various parts of the 3′ end of GAP-43 mRNA and with either a control plasmid, pcDNA3 (Invitrogen), or a plasmid expressing ARPP-19 from a CMV promoter. Twenty-four hours after calcium phosphate-mediated transfection, cells were glycerol-shocked and incubated in the absence or presence of NGF for 3 days. (*a*) Cells were collected and lysed by freeze-thaw. Fluorescence, along with protein concentration, was determined as described in the text. The percent change in fluorescence per μg protein relative to untreated controls is shown for each combination of plasmids in the absence or presence of NGF. (*b*) Single amino acid substitutions of Ser-104 to either an Ala (A) or Asp (D) block the effect of ARPP-19 on reporter stability. (*c*) Similar results were obtained by counting the number of fluorescent cells per well of a 24-well culture plate. (*d*) In the deletion mutant *rbr2*Δ692–764, the combination of NGF plus ARPP-19 failed to stimulate reporter expression. **, Different from NGF-treated cells overexpressing wild-type ARPP-19 and EGFP linked to *rbr2*; $P < 0.01$. ††, Fluorescence suppressed below that of comparable NGF-treated samples expressing endogenous ARPP-19; $P < 0.01$. All assays were carried out in quadruplicate and were blinded.

phosphorylation of recombinant ARPP-19 by PKA did not detectably alter its binding to *rbr2* in gel-shift experiments (data not shown).

Discussion

These studies implicate the phosphoprotein ARPP-19 in the posttranscriptional regulation of GAP-43 expression by NGF. ARPP-19 satisfied the criteria of showing NGF-dependent binding to the 3′ UTR of GAP-43 mRNA and of binding specifically within the 3′ region (*rbr2*) previously shown to confer NGF dependence on the stability of a heterologous reporter construct (17). The association of ARPP-19 with the NGF-regulatory region of the GAP-43 mRNA was demonstrated further through the use of gel-shift assays. Importantly, overexpression of ARPP-19 in PC12 cells conferred NGF-enhanced expression of an EGFP reporter construct that contained the appropriate 3′ region of GAP-43 mRNA. Deletions within the *rbr2* region of GAP-43 mRNA abolished NGF-dependent stabilization. Moreover, basal and NGF-dependent expression of the EGFP reporter was attenuated by mutation of Ser-104 of ARPP-19. This latter effect was associated with a blocking of NGF-mediated neurite outgrowth in transfected PC12 cells.

Regulation of mRNA stability is a major point of control of gene expression. The stability of a wide variety of mRNAs is regulated by the interaction of specific structural elements in the RNA and in mRNA-binding proteins (26). Variations in mRNA half-lives can be detected in response to many developmental and environmental cues, including cytokines, hormones, nutrient levels, temperature changes, hypoxia, viral infection, and tissue damage (27–32). Defects in the control of mRNA stability may be associated with thalassemia and some forms of neoplasia (28). There are two general models of regulated mRNA decay. In deadenylation-dependent decay, the poly(A) tract on the 3′ end of the mRNA is removed by poly(A) ribonuclease (33), the cap at the 5′ end is removed, and the RNA is degraded by exonucleases from both the 3′ and 5′ directions. This pathway leads to the degradation of many short-lived proto-oncogene and cytokine mRNAs that contain A+U-rich elements. An alternative model is the deadenylation-independent pathway, in which endonucleolytic cleavage of the mRNA generates two products that are subject to exonucleolytic digestion (28). The latter pathway leads to the degradation of insulin-like growth factor 2 and transferrin receptor mRNAs. Either of these pathways could be involved in the degradation of GAP-43 mRNA.

Along with *rbr2*, most other regions in the 3′ UTR of GAP-43 mRNA show a high degree of evolutionary conservation and include binding sites for other proteins that contribute to overall stability. We previously identified two proteins that bind to *rbr1* (Fig. 1), a pyrimidine-rich region of GAP-43 mRNA: far upstream sequence element-binding protein (FBP) and a polypyrimidine tract-binding protein (PTB) (19). A third protein that binds to *rbr1* is HuD, a member of the Elav RNA-binding protein family (20, 34). Overexpression of HuD leads to an increase in GAP-43 mRNA stability that is independent of NGF, and reduction of HuD expression using antisense oligonucleotides blocks GAP-43 mRNA stabilization (35). Although the binding of these latter proteins is not NGF-dependent, they may mediate the effects of other signals on GAP-43 mRNA stability (20, 34).

The role of transcriptional control also must be considered in the regulation of GAP-43 expression. In one transgenic mouse line, a reporter linked to 6 kb of sequence upstream of the coding region, the first exon, and 11 kb of the first intron of GAP-43, was found to be expressed in the developing mouse brain in a fashion that closely paralleled the expression pattern of endogenous GAP-43 (36). Others have described putative transcriptional control elements within a 1-kb region 5′ of the transcriptional start site (37, 38). Finally, other *in vivo* studies that compared signals from intronic and exonic probes found that increased levels of GAP-43 that occur after various physiological manipulations were the result of both transcriptional and posttranscriptional mechanisms (39).

ARPP-16 and ARPP-19 first were identified in studies of phosphoproteins in the neostriatum (25). ARPP-16 is highly expressed in medium spiny neurons in the neostriatum. In contrast, ARPP-19 is expressed in all brain regions and is also found in nonneuronal cells. Our further analyses of expressed sequence tag databases have identified ARPP-16/19 homologs in *Caenorhabditis elegans*, *Drosophila melanogaster*, and yeast genomes. Recently, we and others have identified a homolog of ARPP-16/19 (24, 40, 41), which has been termed endosulfine because the protein was purified by using an assay to identify endogenous ligands for the sulfonylurea receptor (42). Endosulfine, like ARPP-19, contains a distinct N terminus attached to a sequence that is very similar to ARPP-16. Because endosulfine differs slightly from ARPP-16 within the core structure, it probably represents a distinct gene product. Together, these observations suggest the existence of an evolutionarily conserved family of ARPP-16/19 phosphoproteins. In addition, a consensus PKA phosphorylation site is found in all family members.

The physiological role of Ser-104 remains to be determined. Mutation of Ser-104 to either Ala or Asp blocked the ability of ARPP-19 to stabilize reporter mRNAs containing *rbr2* at their

3′ ends. However, phosphorylation of recombinant ARPP-19 did not seem to affect its binding to *rbr2* in a gel-shift assay. These results suggest that phosphorylation of Ser-104 by PKA may not be responsible for the NGF-dependent regulation of GAP-43 expression by ARPP-19. Consistent with this conclusion, cAMP has only a modest effect on GAP-43 expression in PC12 cells (43). The observation that mutation of the PKA phosphorylation site in ARPP-19 does not seem to alter its affinity for the *rbr2* region of GAP-43 mRNA, whereas it does alter mRNA stabilization, may imply that Ser-104 is involved in protein–protein interactions that lead to the stabilization of the mRNA. This could be analogous to the activity of the DNA-binding protein CREB, where phosphorylation alters a protein–protein interaction with the CREB-binding protein leading to transcriptional activation (44). In preliminary studies, we have found that ARPP-19 is phosphorylated in intact cells at site(s) that are distinct from the PKA site. In addition, recent *in vitro* studies have indicated that ARPP-16 and ARPP-19 are phosphorylated on a common Ser residue by Cdk5 (unpublished results). Other studies have found that treatment of PC12 cells with NGF leads to increased expression of the p35 regulatory subunit of Cdk5 and to elevated Cdk5 activity (45). Future studies of the phosphorylation of ARPP-19 by Cdk5 therefore might provide a link between NGF and the stabilization of GAP-43 mRNA.

In summary, there is still a great deal to be learned about the mechanisms that control GAP-43 expression *in vivo*. The present studies demonstrate that in PC12 cells, in which the GAP-43 gene already is being transcribed, the phosphoprotein ARPP-19 provides an important link between NGF signaling and structural and functional plasticity through posttranscriptional regulation of gene expression.

We thank Emily Levine, Guang-de Li, and Eugene Alperovich for their excellent assistance and Paul Rosenberg for his critical reading of the manuscript. This work was supported by National Institutes of Health Grants R01 EY05690 (to L.I.B.) and DA10044 (to A.C.N. and P.G.) and by the Boston Neurosurgical Foundation.

1. Aigner, L. & Caroni, P. (1993) *J. Cell Biol.* **123,** 417–429.
2. Shea, T. B., Perrone-Bizzozero, N. I., Beermann, M. L. & Benowitz, L. I. (1991) *J. Neurosci.* **11,** 1685–1690.
3. Strittmatter, S. M., Fankhauser, C., Huang, P. L., Mashimo, H. & Fishman, M. C. (1995) *Cell* **80,** 445–452.
4. Zhang, F., Lu, C., Severin, C. & Sretavan, D. W. (2000) *Development (Cambridge, U.K.)* **127,** 969–980.
5. Maier, D. L., Mani, S., Donovan, S. L., Soppet, D., Tessarollo, L., McCasland, J. S. & Meiri, K. F. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 9397–9402.
6. Aigner, L., Arber, S., Kapfhammer, J. P., Laux, T., Schneider, C., Botteri, F., Brenner, H. R. & Caroni, P. (1995) *Cell* **83,** 269–278.
7. Bomze, H. M., Bulsara, K. R., Iskandar, B. J., Caroni, P. & Skene, J. H. (2001) *Nat. Neurosci.* **4,** 38–43.
8. He, Q., Dent, E. W. & Meiri, K. F. (1997) *J. Neurosci.* **17,** 3515–3524.
9. Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D. & Caroni, P. (2000) *J. Cell Biol.* **149,** 1455–1472.
10. Skene, J. H. (1989) *Annu. Rev. Neurosci.* **12,** 127–156.
11. Benowitz, L. I. & Routtenberg, A. (1997) *Trends Neurosci.* **20,** 84–91.
12. Lovinger, D. M., Colley, P. A., Akers, R. F., Nelson, R. B. & Routtenberg, A. (1986) *Brain Res.* **399,** 205–211.
13. Routtenberg, A., Cantallops, I., Zaffuto, S., Serrano, P. & Namgung, U. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7657–7662.
14. Perrone-Bizzozero, N. I., Cansino, V. V. & Kohn, D. T. (1993) *J. Cell Biol.* **120,** 1263–1270.
15. Perrone-Bizzozero, N. I., Neve, R. L., Irwin, N., Lewis, S., Fischer, I. & Benowitz, L. I. (1991) *Mol. Cell Neurosci.* **2,** 402–409.
16. Federoff, H. J., Grabczyk, E. & Fishman, M. C. (1988) *J. Biol. Chem.* **263,** 19290–19295.
17. Nishizawa, K. (1994) *Biochem. Biophys. Res. Commun.* **200,** 789–796.
18. Neve, R. L., Ivins, K. J., Tsai, K. C., Rogers, S. L. & Perrone-Bizzozero, N. I. (1999) *Mol. Brain Res.* **65,** 52–60.
19. Irwin, N., Baekelandt, V., Goritchenko, L. & Benowitz, L. I. (1997) *Nucleic Acids Res.* **25,** 1281–1288.
20. Chung, S., Eckrich, M., Perrone-Bizzozero, N. I., Kohn, D. T. & Furneaux, H. (1997) *J. Biol. Chem.* **272,** 6593–6598.
21. Houman, F., Diaz-Torres, M. R. & Wright, A. (1990) *Cell* **62,** 1153–1163.
22. Girault, J. A., Horiuchi, A., Gustafson, E. L., Rosen, N. L. & Greengard, P. (1990) *J. Neurosci.* **10,** 1124–1133.
23. Leibolb, E. A. & Munro, H. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2171–2175.
24. Dulubova, I., Horiuchi, A., Snyder, G. L., Girault, J. A., Czernik, A. J., Shao, L., Ramabhadran, R., Greengard, P. & Nairn, A. C. (2001) *J. Neurochem.* **77,** 229–238.
25. Horiuchi, A., Williams, K. R., Kurihara, T., Nairn, A. C. & Greengard, P. (1990) *J. Biol. Chem.* **265,** 9476–9484.
26. Ross, J. (1995) *Microbiol. Rev.* **59,** 423–450.
27. Brennan, C. M. & Steitz, J. A. (2001) *Cell. Mol. Life Sci.* **58,** 266–277.
28. Guhaniyogi, J. & Brewer, G. (2001) *Gene* **265,** 11–23.
29. Staton, J. M., Thomson, A. M. & Leedman, P. J. (2000) *J. Mol. Endocrinol.* **25,** 17–34.
30. Paulding, W. R. & Czyzyk-Krzeska, M. F. (2000) *Adv. Exp. Med. Biol.* **475,** 111–121.
31. Conne, B., Stutz, A. & Vassalli, J. D. (2000) *Nat. Med.* **6,** 637–641.
32. Mitchell, P. & Tollervey, D. (2000) *Curr. Opin. Genet. Dev.* **10,** 193–198.
33. Korner, C. G. & Wahle, E. (1997) *J. Biol. Chem.* **272,** 10448–10456.
34. Tsai, K. C., Cansino, V. V., Kohn, D. T., Neve, R. L. & Perrone-Bizzozero, N. I. (1997) *J. Neurosci.* **17,** 1950–1958.
35. Mobarak, C. D., Anderson, K. D., Morin, M., Beckel-Mitchener, A., Rogers, S. L., Furneaux, H., King, P. & Perrone-Bizzozero, N. I. (2000) *Mol. Biol. Cell* **11,** 3191–3203.
36. Vanselow, J., Grabczyk, E., Ping, J., Baetscher, M., Teng, S. & Fishman, M. C. (1994) *J. Neurosci.* **14,** 499–510.
37. Udvadia, A. J., Koster, R. W. & Skene, J. H. (2001) *Development (Cambridge, U.K.)* **128,** 1175–1182.
38. Eggen, B. J., Nielander, H. B., Rensen-de Leeuw, M. G., Schotman, P., Gispen, W. H. & Schrama, L. H. (1994) *Mol. Brain Res.* **23,** 221–234.
39. Namgung, U. & Routtenberg, A. (2000) *Eur. J. Neurosci.* **12,** 3124–3136.
40. Peyrollier, K., Heron, L., Virsolvy-Vergine, A., Le Cam, A. & Bataille, D. (1996) *Biochem. Biophys. Res. Commun.* **223,** 583–586.
41. Virsolvy-Vergine, A., Salazar, G., Sillard, R., Denoroy, L., Mutt, V. & Bataille, D. (1996) *Diabetologia* **39,** 135–141.
42. Virsolvy-Vergine, A., Leray, H., Kuroki, S., Lupo, B., Dufour, M. & Bataille, D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6629–6633.
43. Costello, B., Meymandi, A. & Freeman, J. A. (1990) *J. Neurosci.* **10,** 1398–1406.
44. Shaywitz, A. J., Dove, S. L., Kornhauser, J. M., Hochschild, A. & Greenberg, M. E. (2000) *Mol. Cell. Biol.* **2,** 9409–9422.
45. Harada, T., Morooka, T., Ogawa, S. & Nishida, E. (2001) *Nat. Cell Biol.* **3,** 453–459.