Differential Proteomics Reveals Multiple Components in Retrogradely Transported Axoplasm After Nerve Injury*

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Information on axonal damage is conveyed to neuronal cell bodies by a number of signaling modalities, including the post-translational modification of axoplasmic proteins. Retrograde transport of a subset of such proteins is thought to induce or enhance a regenerative response in the cell body. Here we report the use of a differential 2D-PAGE approach to identify injury-correlated retrogradely transported proteins in nerves of the mollusk Lymnaea. A comprehensive series of gels at different pI ranges allowed resolution of ~4000 proteins by silver staining, and 172 of these were found to differ between lesioned versus control nerves. Mass spectrometric sequencing of 134 differential spots allowed their assignment to over 40 different proteins, some belonging to a vesicular ensemble blocked by the lesion and others comprising an up-regulated ensemble highly enriched in calpain cleavage products of an intermediate filament termed RGP51 (retrograde protein of 51 kDa). Inhibition of RGP51 expression by RNA interference inhibits regenerative outgrowth of adult Lymnaea neurons in culture. These results implicate regulated proteolysis in the formation of retrograde injury signaling complexes after nerve lesion and suggest that this signaling modality utilizes a wide range of protein components. Molecular & Cellular Proteomics 3:510–520, 2004.

Injured neurons from mammalian peripheral or invertebrate central nerves retain a capacity for functional regeneration that is dependent on extrinsic cues as well as on factors intrinsic to the cell (1–3). The latter have been postulated to include injury signals emanating from the lesion site of the damaged axons. Such injury signals may include axoplasmic proteins activated by post-translational modifications at the injury site and conveyed by retrograde transport to the cell body (4, 5). Placement of ligatures between the neuronal cell bodies and the lesion site in an injured nerve allows the collection of axoplasm enriched in retrogradely transported components, and studies by Amborn and colleagues in the mollusk Aplysia have sought to identify such components by a candidate protein approach (6). The identity of most Aplysia retrograde injury signaling proteins has not yet been determined, although at least one appears to belong to the mitogen-activated protein kinase family (7, 8). An importin-dynein complex was recently shown to transport retrograde injury signal proteins in mammalian nerve, but the identity of these signaling proteins is still unknown (9).

The candidate protein approach can occasionally be very successful, but the anatomical and molecular complexity of the nervous system suggest that most signaling systems are not likely to be critically dependent on single proteins (10). Thus for example the protein complexes involved in synaptic signal transduction are typically made up of ~100 interacting proteins, and most recent estimates suggest that 700–1000 proteins are required to create the signaling network for a working synapse (10–12). It is very likely that long-range retrograde transduction of signals along axons will require molecular machines of similar complexity in composition. We therefore sought to characterize retrograde injury signal complexes by using a comprehensive differential proteomics approach in the freshwater mollusk Lymnaea stagnalis. This invertebrate model nervous system is advantageous for both in vitro and in vivo analyses of regeneration in single neurons and small networks (13–15), and both Lymnaea and other mollusks have served as pioneer preparations for development of proteomic approaches in the nervous system (16–18). We used two-dimensional electrophoresis to compare retrogradely concentrated axoplasm from lesioned and control Lymnaea nerve and identified differential spots by high-performance tandem mass spectrometry (MS). The results indicate that retrograde injury signaling may be mediated by soluble protein complexes arising from cleavage or modification of a protein.

1 The abbreviations used are: MS, mass spectrometry; 2D-PAGE, two-dimensional PAGE; CID, collision-induced dissociation; DTT, di-thiothreitol; HRP, horseradish peroxidase; IF, intermediate filament; IPG, immobilized pH gradient; LPS, Lymnaea physiological saline; RACE, rapid amplification of cDNA ends; RGP, retrograde protein; dsRNA, double-stranded RNA.
A wide variety of axonal proteins coupled with the loss of vesicular signal components normally trafficked in the nerve.

**EXPERIMENTAL PROCEDURES**

**Biological System**—Central ganglia of *Lymnaea* were dissected to expose the main nerve tracts radiating to peripheral targets. Nerves were first ligated close to the ganglia and then crush-lesioned with fine forceps 2 cm from the ligation site. The animals were incubated in a bath of *Lymnaea* physiological saline (LPS: NaCl 53 mM, KCl 1.7 mM, CaCl₂ 4.1 mM, MgCl₂ 1.5 mM, HEPES 5.0 mM, pH 7.9) at 20 °C, and at given time points axoplasm was collected from ligated nerve segments by gentle squeezing into clean sterilized LPS with 1 mM orthovanadate and protease inhibitors mixture (Merck, West Point, PA). The extract was clarified by a 1-min centrifugation at 20,000 g to obtain pellet 1. The supernatant was then subjected to ultracentrifugation for 1 hour at 250,000 g to obtain pellet 2 and soluble fractions. Both pellets were solubilized separately in lysis buffer with proteinase and phosphatase inhibitors. The three extracts were then processed separately for two-dimensional PAGE (2D-PAGE) analysis.

**2D-PAGE**—Aliquots of 250–750 μg of protein were precipitated with 10% trichloroacetic acid in acetone and 20 mM dithiothreitol (DTT), and protein pellets were resuspended in rehydration solution (7 M urea, 2 M thiourea, 2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 60 mM DTT, 2% Phormalyte). First-dimension isoelectric focusing was carried out on an IPGphor system (Amersham Biosciences, Piscataway, NJ) essentially as described by the manufacturer. Preacast immobilized pH gradient (IPG) strips (18 cm; 3.5–4.5, 4.5–5.5, 5–6, 5.5–6.7, 4–7, 3–10, 6–11) were used in first-dimensional separation for a total of 35–70 kV·h. After the first-dimension separation, the strips were equilibrated with a solution containing 6 M urea, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 1% DTT, and bromophenol blue for 15 min. A second equilibration was then performed in DTT-free solution containing 4% iodoacet-

![Image](image_url)
amide for 15 min. The strips were then directly applied to 10% Duracryl high-tensile strength acrylamide gels (Genomic Solutions, Ann Arbor, MI) for electrophoresis. Strips were overlaid with 0.5% low-melting-point agarose in running buffer containing bromophenol blue. Gels were run in a Hoefer DALT system (Amersham Pharmacia Biotech, Uppsala, Sweden), fixed and stained by a mass spectrometry-compatible silver stain, and then scanned and analyzed using ImageMaster 2D Elite software (Amersham Pharmacia Biotech) or Z3 software (Compugenue, St. Louis, MO). Gels were always run in duplicate, and each experiment was repeated at least three times. Protein spots revealing over 3-fold change (after normalization) between lesion and control samples were excised and digested in-gel with trypsin (donatello.ucsf.edu/ingel.html).

**Tandem Mass Spectrometry**—The digests were analyzed by liquid chromatography MS/MS using an Ultimate high-performance liquid chromatography system equipped with a FAMOS autosampler and a C18 PepMap 75-μm × 150-mm column (Dionex-LC-Packings, San Francisco, CA). Solvent A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile, at a flow rate of 350 nl/min. Approximately one-tenth of each digest (1 μl) was injected at 5% B, then the organic content of the mobile phase was increased linearly to 50% over 30 min. The column effluent was directed to a quadrupole-orthogonal-acceleration-time-of-flight QSTAR Pulsar tandem mass spectrometer equipped with a Microlonspray source (Applied Biosystems/MDS Sciex, Toronto, Ontario, Canada). Throughout the analysis, 1-s MS acquisitions were followed by 5-s collision-induced dissociation (CID) experiments in information-dependent acquisition mode, i.e. multiply charged ions fulfilling certain preset criteria were selected by the computer as precursor ions, and the most common trypsin autolysis products were excluded. The collision energy was set according to the mass value and charge state of the precursor ion. The CID spectra were interpreted manually.

**Bioinformatics**—Because no mollusk genome has yet been sequenced, protein assignment was carried out with the aid of database homology search engines (19) such as MS-Pattern (www.prospector.ucsf.edu) and MS-Blast (dove.embl-heidelberg.de/Blast2/msblast.html).

**Cloning of RGP51**—Degenerate primers comprising all possible codons for the sequences LEAENEA and AAHIEADC (primer T <58°C) were used for PCR on cDNA from Lymnaea central nervous system. A single PCR product of 400 bp was cloned and sequenced. Specific primers based on this sequence (5'-GATGACTTACGGGCGCTG and 3’-AAACGCCAAGCTCCGAGAAC, Tm of 55°C) were then used to obtain a full-length cDNA by 5' and 3' RACE (Roche Molecular Biochemicals, Indianapolis, IN). Alignments and trees of RGP51 and its closest homologs were constructed using ClustalX.

**RNA Interference**—PCR was conducted to obtain a 1039-bp RGP51 cDNA fragment flanked on both strands with the T7 promoter. The PCR product was then used as a template for transcription of double-stranded RNA (dsRNA) using T7 RNA Polymerase (Roche Molecular Biochemicals). The dsRNA was applied to a final concentration of 10 pm in cultures of L. stagnalis PedalA neurons as previously described (20). Cultured neurons were incubated in a dark humidified chamber for up to 72 h and scored for neurite sprouting under an inverted microscope (Axiovert 135; Zeiss, Thornwood, NY). Neurons were considered to have sprouted if they contained at least 3 neurite branches.
1 neurite with distinct growth cones and filopodia that were at least 2 soma diameters in length.

RESULTS

Nerve tracts radiating outwards from the central ganglia of adult *Lymnaea* were exposed, ligated close to the ganglia, and incubated with or without nerve crush in a bath of physiological saline at 20 °C. Retrograde transport rates in the system were estimated to be in the range of 1–1.2 mm/h by horseradish peroxidase (HRP) tracer microinjection (Fig. 1). Because crush sites in the nerves were on average 2 cm distant from the corresponding ganglia, the 18- to 24-h time window was selected for collection of axoplasm at the ligation sites. Axoplasm underwent fractionation into three pools as illustrated in Fig. 1, comprising relatively less soluble components obtained from initial low-speed centrifugation (pellet 1) and vesicular and endosomal components arising from ultracentrifugation (pellet 2) and the soluble components not precipitated by either centrifugation step. All three pools were fractionated on large format 18/11003/20-cm 2D-SDS-PAGE gels comparing control to lesion samples (Fig. 2). In order to obtain the most comprehensive characterization possible, we used narrow-range IPG strips wherever necessary, allowing increased resolution and higher protein loads (Fig. 3). Eight different combinations of pI and molecular mass ranges were run to resolve a total of 4000 axoplasmic protein spots by silver staining, 172 of which were clearly differentially represented in lesioned versus control axoplasm. Tryptic digests of the differential spots followed by de novo peptide sequencing using low-energy CID spectra resulted in peptide sequences from a total of 134 spots that could be assigned by homology to over 40 different proteins with diverse cellular functions (Table I). Different post-translational modifications in some of

**Table I**

| Category                  | Identity                        | Control | Injured |
|---------------------------|---------------------------------|---------|---------|
| Cytoskeleton              | Intermediate filament           | 2       | 13      |
|                           | Actin                           | 2       | 16      |
|                           | Tubulin                         | 1       | 1       |
|                           | Myosin                          | 4       | –       |
|                           | Tropomyosin                     | –       | 2       |
|                           | Actin modulator                 | –       | 2       |
|                           | Matrilin                        | 1       | –       |
|                           | Myoglobin                       | –       | 1       |
| Glycolysis                | Triose-phosphate isomerase      | 1       | –       |
|                           | Enolase (2-phosphoglycerate dehydratase) | 1 | –       |
|                           | GAPDH                           | –       | 1       |
| Redox                     | Reductase-related protein       | 1       | –       |
|                           | Peroxisorixin                   | –       | 2       |
|                           | Cytochrome B5                   | 2       | –       |
|                           | Pyrroline-5-carboxylase reductase | – | 1       |
| Kinase                    | Phosphoglycerate kinase (PGK)   | –       | 2       |
|                           | Adenylate kinase                | –       | 1       |
|                           | Arginine kinase                 | 2       | –       |
| Detoxification            | Glutathione S-transferase       | 1       | –       |
|                           | Tellurium resistance protein    | –       | 1       |
|                           | Ferritin                        | 2       | –       |
| Chaperones                | HSP60                           | –       | 1       |
|                           | Cyclophilin                     | –       | 2       |
|                           | 14-3-3                          | 2       | –       |
| Calcium binding           | Calbindin                       | 1       | –       |
|                           | Calmodulin                      | 2       | –       |
| Ubiquitin                 | Ubiquitin carboxyl-terminal hydrolase (ubiquitin thiolesterase) | 4 | 4       |
| Membrane                  | Voltage-dependent anion channel | 1       | –       |
|                           | Synaptotagmin                   | 5       | –       |
| Metabolic                 | Glutamine synthetase            | –       | 1       |
|                           | ATP synthase                    | 1       | 2       |
|                           | Argininosuccinate synthase      | –       | 1       |
|                           | Protein disulfide isomerase     | 2       | 1       |
| RNA synthesis             | Ribosomal protein L7            | –       | 1       |
|                           | RNA polymerase B transcription factor 3(BTF3) | 1 | –       |
| Others                    | Schistosomin                    | –       | 1       |
|                           | Acetycholine-binding protein    | 2       | 1       |
|                           | Proteasome subunit              | 1       | –       |
|                           | RHO GDP dissociation inhibitor   | 1       | –       |
| Unknown                   | No assignable homology          | 12      | 21      |

* The peptide sequences used for protein assignment are provided in the supplemental tables on-line.

1 neurite with distinct growth cones and filopodia that were at least 2 soma diameters in length.


### TABLE II

| Modification     | Protein                  | Predicted mass (kDa)<sup>a</sup> | Apparent mass on gel (kDa) |
|------------------|--------------------------|-----------------------------------|---------------------------|
|                  |                          | Control                          | Injured                   |
| Proteolysis      | Intermediate filament    | 51                                | 16, 27, 36                |
| Actin            | 42                       | 15<sup>b</sup>                    | 10, 15<sup>b</sup>        |
| Adenylate kinase | 18                       | –                                 | 9.5                       |
| Protein disulfide isomerase | 57                      | 17, 25                            | –                         |
| Reductase-related protein | 51                       | 23                                | –                         |
| Ubiquitin carboxyl-terminal hydrolase | 24           | –                                 | 14                        |
| Yolk ferritin precursor | 27                      | 10                                | –                         |
| Peroxiredoxin   | 22                       | –                                 | 10                        |
| Calbindin       | 35                       | 26                                | –                         |
| Acetylcholine-binding protein | 26                     | 13                                | –                         |
| Glutathione S-transferase | 25                   | 11                                | –                         |
| Synaptotagmin   | 48                       | 30                                | –                         |
| Increased M<sub>i</sub> | Intermediate filament | 51                                | 100                       |
| Actin            | 42                       | –                                 | 101, 120                  |
| Tubulin          | 50                       | –                                 | 110                       |
| Tropomyosin      | 33                       | –                                 | 63                        |
| ATP synthase     | 54                       | –                                 | 117                       |
| Phosphoglycerate kinase | 44                     | –                                 | 112                       |
| HSP60            | 62                       | –                                 | 105                       |
| Ubiquitin carboxyl-terminal hydrolase | 24           | –                                 | 60, 90                    |
| Acetylcholine-binding protein | 26                     | –                                 | 43                        |
| Arginine kinase  | 39                       | 85                                | –                         |
| Enolase          | 47                       | 70                                | –                         |
| Synaptotagmin    | 48                       | 104                               | –                         |
| Actin modulator  | 41                       | –                                 | 113, 122                  |
| Matrilin         | 44                       | 100                               | –                         |

<sup>a</sup> Predicted mass is according to the corresponding *Lymnaea* protein or the closest homolog in the databases.

<sup>b</sup> The two approximately 15-kDa actin fragments are from different regions in the protein (Fig. 7).

### TABLE III

| Fraction | Control | Injured |
|----------|---------|---------|
| Pellet 1 | 2       | Intermediate filament, actin, tubulin, tropomyosin, ribosomal protein L7, ATP synthase, phosphoglycerate kinase, tellurium resistance protein, peroxiredoxin, HSP60, glutamine synthetase RGP55, RGP19, RGP62, RGP120, RGP121 RGP57, RGP118 |
|          | 42      | Intermediate filament |
| Pellet 2 | 24      | Intermediate filament |
| Soluble | 29      | Myosin, protein disulfide isomerase, tubulin, enolase, ATP synthase, calmodulin, cytochrome B5, ubiquitin carboxyl-terminal hydrolase, 14-3-3, matrilin, reductase-related protein, arginine kinase, acetylcholine-binding protein, RGP30, RGP28, RGP15, RGP20, RGP70 |
|          | 35      | Actin, tropomyosin, myoglobin, GAPDH, schistosomin, peroxiredoxin, cyclophilin, adenylate kinase, acetylcholine-binding protein, protein disulfide isomerase, phosphoglycerate kinase, actin modulator, argininosucinate synthase, ubiquitin carboxyl-terminal hydrolase, pyrroline-5-carboxylase reductase, RGP12, RGP34, RGP43, RGP11, RGP10, RGP25, RGP9, RGP13, RGP20, RGP37, RGP97, RGP100 |

<sup>a</sup> The numbers refer to the number of differential assignments for each fraction and category. Spot numbers may differ from the number of protein identifications, due to different isoforms or modifications of a single protein appearing in multiple spots; or to more than one protein identified in a single spot. RGP indicates retrograde protein, specified according to apparent mass on gel, thus for example RGP70 stands for retrograde protein of apparent 70 kDa. This nomenclature is used for spots containing peptide sequences with no assignable homology in the databases.
FIG. 4. Low-energy CID spectra of two tryptic peptides providing the sequences used in the cloning of the IF protein. Peptide fragments are labeled according to Biemann’s nomenclature (42). The identity of isomeric Ile/Leu residues cannot be determined from these data. Those were assigned based on homology to IF proteins from other species and eventually from the cDNA sequence (see Fig. 5). The accuracy of the mass measurements afforded by the QSTAR mass spectrometer permits the differentiation between isobaric Gln and Lys residues.
these proteins were revealed by MS, including N-terminal acetylation and glycosylation (data not shown) and a widespread novel sulfonation of threonine and serine residues (21). In addition to these directly determined modifications, many of the spots contain proteolytic cleavage fragments of the identified proteins or conversely variants with increased mass that may represent ubiquitinated or cross-linked species (Table II).

Categorization of the identified proteins according to the axoplasm prefractionation procedure (Table III) reveals that the differential proteins in pellet 2 were primarily down-regulated in axoplasm from lesioned nerves. Pellet 2 was obtained by an ultracentrifugation procedure designed to isolate membranous vesicles from the axoplasm, and indeed a number of the components in this fraction are membrane proteins, including a voltage-dependent anion channel and synaptotagmin. These results indicate that vesicular components that normally traffic retrogradely in the nerve are blocked by the lesion, thus potentially providing a down-regulated signal to the cell body.

The two most prominent proteins identifiable by homology search on the peptide sequences were from the intermediate filament (IF) and actin families (Table I). The possibility that these represent a trivial consequence of cytoskeletal destruction after the crush is not likely, because other cytoskeleton components known to be highly abundant in axons, such as neurofilament or spectrin, were not found in any of the spots analyzed. Peptide sequences from the IF spots were used to design degenerate primers for PCR and subsequent cloning of the complete cDNA (Figs. 4 and 5). All IF-like spots were found to originate from a single gene product, designated RGP51 (retrograde protein 51 kDa). RGP51 reveals the typical secondary structure of the IFs (22), and the spot analyses indicate that the major RGP51 components up-regulated by lesion are cleavage products of apparent masses 16, 27, and 36 kDa (Figs. 6 and 7). Similarly, the main actin spots up-regulated by lesion are cleavage products, in this case of 10 and 15 kDa (Fig. 7). Peptide sequences determined from the mass spectrometric analyses enable mapping of the various cleavage products on the complete protein sequence and determination of one of the cleavage sites in RGP51 (Fig. 7). This cleavage site does not match any specific proteolytic consensus motif. However, the calcium-activated protease calpain is known to be activated upon nerve injury and has been linked to the proteolytic degradation of cytoskeletal proteins (23–25). We therefore conducted in vitro cleavage assays of 35S-RGP51 produced by in vitro translation and incubated with axoplasm from injured Lymnaea nerve. As shown in Fig. 7C, 35S-RGP51 was cleaved in axoplasm, and the cleavage was blocked in the presence of the calcium chelator EGTA or the calpain inhibitor, calpeptin. The apparent masses of the two cleavage products obtained in this experiment correspond well with the endogenous fragments observed in the 2D-PAGE screen.

In order to assess the functional significance of RGP51 for neuronal regeneration in Lymnaea, we carried out dsRNA-mediated knock-down of RGP51 expression in cultures of axotomized adult Lymnaea neurons. As shown in Fig. 8, RGP51-depleted neurons did not regenerate in culture over a 72-h period, whereas we observed robust sprouting of control neurons. The RGP51 dsRNA treatment did not affect the gross morphology of the neuronal cell bodies and did not affect their survival over the time course of the experiment (data not shown). Thus, a molluscan axonal IF protein undergoes cleavage and retrograde transport upon nerve lesion and is necessary for an appropriate regenerative response by lesioned neurons.

**DISCUSSION**

The objective of this study was to obtain a comprehensive overview of the retrogradely transported protein ensemble after nerve injury by using 2D-PAGE to compare control versus lesion axoplasm retrogradely concentrated at nerve ligations. Although many laboratories are studying differential gene expression after nerve injury, most such work is at transcriptome level (26, 27) and does not address the translational and post-translational regulation thought to occur in...
Fig. 6. A, low-energy CID spectrum of the N-terminal peptide from tryptic digests of the 36- and 27-kDa fragments of RGP51, establishing Ser-58 as the N terminus for these fragments. B, low-energy CID spectrum of the predicted C-terminal tryptic peptide of RGP51.
Lesioned axons (5). Studies to date on retrograde injury signaling proteins in lesioned nerve have mainly focused on candidate proteins selected according to prior knowledge of their signaling or transcriptional effects in other systems, resulting in the implication of two kinases in this process (7, 8). Although some of the novel proteins found in our screen contain characteristics of kinase-regulated signaling proteins, the results implicate a much broader range of components in retrograde injury signaling (Table I and supplemental on-line data). These include a “negative injury signal” vesicular ensemble comprising proteins normally transported in axons that are down-regulated by the lesion (Table III), perhaps due to the physical barrier preventing normal passage of vesicles originating at the nerve terminal. In addition to this vesicular component, numerous other proteins were identified in soluble axoplasm and provide new candidate signaling proteins for the process. These additional proteins appear to arise from a number of post-translational modifications, the most prevalent of which are changes in the apparent mass of the modified proteins, including mass increases that may be due to multimerization or ubiquitination, and mass decreases that are indicative of proteolysis in the axoplasm (Table II).

One of the most prominent of the identified proteins in our screen was the IF homolog termed RGP51, various forms of which were identified in nearly 25% of the differential spots. Cloning of a bona fide *Lymnaea* cDNA encoding this protein confirmed nearly all mass spectrometric sequence assignments, and the combination of MS and cloning data allowed definitive identification of a cleavage site that gives rise to the N-terminal of two major fragments of RGP51 in injury axoplasm. Because this cleavage site does not correspond to any obvious specific protease consensus, we examined the possible involvement of the calcium-activated protease calpain. Calpain is known to be activated upon nerve injury (23) and to cleave its substrates at a restricted number of sites that are most likely conformational rather than sequence specific (28). Axoplasmic cleavage of *in vitro*-translated RGP51 was blocked by calcium chelation with EGTA or by calpeptin, a calpain-specific blocker. Thus, calpain activity generates at least some of the IF products transported retrogradely upon nerve injury, and may also underlie the concomitant shift in actin products. Although calpain has been implicated in pathological processes after nerve trauma (29, 30), selective activation of calpain is not necessarily detrimental. In fact transient calpain activity is required for specific events in neuronal plasticity and regeneration, such as dendritic remodeling (24), membrane sealing (31, 32), and growth cone formation and

**Fig. 7.** Cleavage maps of RGP51 (A) and actin (B) showing positions of major cleavage products observed on gel on a secondary structure schematic of the full protein. The C-terminal 15-kDa fragment of actin is down-regulated by nerve lesion, all other indicated fragments from both proteins are up-regulated by the injury. Positions of the peptide sequences obtained from MS are indicated underneath each fragment, these peptides served to anchor the fragments on the cleavage map. The N-terminal sequence of the 27- and 36-kDa fragments of RGP51 was determined by MS/MS (see Fig. 6A), the inferred cleavage site is therefore indicated by an arrow. C, *in vitro* cleavage of 35S-labeled RGP51 upon incubation in axoplasm. Addition of EGTA (100 mM) or of calpeptin (100 μM) inhibits the cleavage activity.

**Fig. 8.** Inhibition of RGP51 expression by RNA interference in cultured adult *Lymnaea* PedalA neurons causes a significant reduction in the regenerative outgrowth of the cells after 72 h in culture. Scale bar, 50 μm.
guidance (25, 33). These studies suggest that calpain activation may promote a local spectrin-dependent reorganization at the lesion site that enhances membrane sealing and growth cone formation (34). Our data now indicates that an additional role for calpain activation at the lesion site might be the cleavage of accessory cytoskeletal substrates other than spectrin to enable targeting of the appropriate fragments to the retrograde transport pathway.

Once targeted to the retrograde transport pathway, what might be the potential signaling function of the identified intermediate filament fragments? So far candidate retrograde injury signaling molecules have mostly been thought to be kinases (8), thus the proteomic approach points out unexpected directions. We used dsRNA inhibition to confirm that RGP51 is required for regeneration of adult Lymnaea PedalA neurons in vitro. Since tubulin and neurofilament are the major structural components of the axonal cytoskeleton, the dsRNA inhibition result supports a critical role for RGP51 in signaling events underlying regeneration. Nonetheless it should be noted that we cannot rule out a structural role for RGP51 in axon initiation or elongation. The dichotomy between structural (35) versus signaling (36, 37) functions of IF is a recurrent theme in the literature and indeed both aspects may be reflected in our dataset. Mammalian IF multimers were shown to move retrogradely after cytoskeleton collapse, translocating from an extended network to a perinuclear distribution in transfected cells (38). The high-molecular-mass species observed on our gels may reflect such a structural redistribution in the axon. On the other hand, IF monomers or truncation products may act as scaffolds for the transport of signaling proteins such as cytosolic phospholipase A2 (39) or may influence signaling or transcription by direct binding to downstream effectors or to DNA (40, 41). Future work will be needed to examine such possibilities for IF cleavage products in axonal retrograde signaling after nerve injury. To summarize, our results implicate regulated proteolysis in the formation of retrograde injury signaling complexes after nerve lesion and suggest that this signaling modality utilizes a wide range of protein components.

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