Characterization of New Cell Permeable C3-Like Proteins That Inactivate Rho and Stimulate Neurite Outgrowth on Inhibitory Substrates

Matthew J. Winton§, Catherine I. Dubreuil§, Dana Lasko‡, Nicole Leclerc§, and Lisa McKerracher§¶.

§Département de Pathologie et biologie cellulaire, Université de Montréal, Montréal Québec, H3T 1J4, ‡BioAxone Thérapeutique Inc., 2900 Édouard Montpetit, P-906 Pavillon Principal, Montréal, Québec, H3T 1J4, Canada.

Running Title: New C3-like Chimeric Proteins

¶To whom correspondence should be addressed:

Université de Montréal, Dépt. de Pathologie et biologie cellulaire
2900 Édouard Montpetit, Pavillon Principal, S-507,
Montréal, Québec, Canada, H3T 1J4.
Tel: 514-343-6111 ext. 1472; Fax: 514-343-5755;
E-mail: mckerral@patho.umontreal.ca

Copyright 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
SUMMARY

The activation state of Rho is an important determinant of axon growth and regeneration in neurons. Axons can extend neurites on growth inhibitory substrates when Rho is inactivated by C3-ADP-ribosyltransferase (C3). We found by Rho-GTP pull-down assay that inhibitory substrates activate Rho. To inactivate Rho scrape loading of C3 was necessary, as it does not freely enter cells. To overcome the poor permeability of C3, we made and characterized five new recombinant C3-like chimeric proteins designed to cross the cell membrane by receptor-independent mechanisms. These proteins were constructed by the addition of short transport peptides to the carboxyl terminal of C3 and tested using a bioassay measuring neurite outgrowth of PC-12 cells plated on growth inhibitory substrates. All five constructs stimulated neurite outgrowth, but with different dose-response profiles. Biochemical properties of the chimeric proteins were examined using C3-05, the most effective construct tested. Gel shift assays showed that C3-05 retained the ability to ADP-ribosylate Rho. Western blots and immunocytochemistry were used to verify the presence of C3 inside treated cells. C3-05 was also effective at promoting neurite outgrowth in primary neuronal cultures, as well as causing the disassembly of actin stress fibers and focal adhesions complexes in fibroblasts. These studies demonstrate that the new C3-like proteins are effective in delivering biologically active C3 into different cell types, thereby, inactivating Rho.
INTRODUCTION

Rho GTPase regulates the actin cytoskeleton and cell motility in response to extracellular signals. Initial studies using Swiss 3T3 fibroblasts demonstrate the ability of Rho to regulate the formation of actin stress fibers and focal adhesion complexes in non-neuronal cells (1). In neurons, Rho plays a key role in determining the response of axons to growth inhibitory proteins. GTPases have two conformations: a GDP-bound inactive state and a GTP-bound active state (2). The activation of Rho in neurons causes growth cone collapse, neurite retraction and cell body rounding (3-5). Treatment with C3-ADP-ribosyltransferase (C3)$^1$, a specific inhibitor of Rho, stimulates axon growth and regeneration (6,7). To be effective, this 24 kDa protein must cross the plasma membrane and interact with intracellular Rho, however, C3 does not easily enter cells. To date, various methods have been used to help facilitate the entry of C3 into cells. In experiments using fibroblasts, C3 is microinjected into individual cells (1), whereas in studies using neuronal cells, triturating (8), or scrape loading techniques (7) are used to aid cellular entry. The need for such disruptive methods to inactive Rho by C3, and the inability to treat all cell types with equivalent techniques has limited the use of C3 as a tool for biochemical studies on Rho signalling. One solution has been to create a fusion protein that increases the efficiency of C3 delivery across the membrane. One such protein, a fusion between C3 and the B subunit of diphtheria toxin (DT), binds to cell surface DT receptors and is internalized by an endocytosis-mediated mechanism (9). This fusion protein is only effective in cells that contain DT receptors, therefore excluding most rodent cells (9). A fusion protein between the bacterial toxin C2 and C3-transferase, C2IN-C3, also increases the ability of C3 to cross the cell membrane by
C3-like Chimeric Proteins

receptor-mediated endocytosis (10). However, C2IN-C3 cannot independently cross the cell membrane because it requires the presence of the C2 toxin-binding component, C2II, to enter cells (10,11). Both of these fusion proteins enter cells by receptor-mediated endocytotic pathways, and therefore, may be trapped within vesicles, which may lessen efficient interaction with Rho.

Small peptides can act as carriers by transporting large protein cargo across cell membranes. Such peptides are part of larger proteins that are able to cross biological membranes. A series of different classes of transport peptides exist: 1) the human immunodeficiency virus transcription activator (Tat) contains a region spanning amino acids 37 to 72, which translocates its cargo to both the cytosol and nucleus (12,13). A shorter Tat sequence, spanning amino acids 48 to 60, is also effective (14). 2) The third helix of the Antennapedia homeodomain (Antp), a Drosophila homeoprotein, possesses the ability to cross biological membranes. Experiments using biotinylated forms of this 16 amino acid peptide have confirmed its ability to penetrate cells and locate in both the cytosol and nucleus (15-17). 3) Prolines are functional participants in some transport peptides (18). Proline residues act as helix breakers and form turn structures within peptides. Peptides rich in proline can form conformations that help in membrane translocation (19). 4) Peptides of 7 amino acids in length or longer that contain basic, arginine-rich sequences can act as effective transport peptides (20). 5) The hydrophobic regions of several membrane transport sequences (MTS) can translocate across the cellular membrane and accumulate in the nucleus (21). 6) Recently, a short amphipathic peptide carrier, Pep-1, was also shown to be able to translocate across cellular membranes (22). We have made and tested four different classes of transport peptides:
Tat (C3-02, C3-03); Antennapedia homeodomain (C3-04); a proline-rich fusogenic peptide sequence (C3-05); and a highly basic, arginine-rich sequence corresponding to the reverse Tat sequence (C3-06). We provide evidence that these five new C3-like chimeric proteins all cross the plasma membrane, inactivate Rho, and stimulate neurite outgrowth on inhibitory substrates.

EXPERIMENTAL PROCEDURES

Materials — The pGEX-2T and pGEX-4T vector systems were obtained from Amersham Pharmacia (Baie d’Urfé, Québec, Canada). Oligonucleotides were purchased from Gibco Life Technologies (Burlington, Ontario, Canada), polymerase chain reaction (PCR) was carried out using a DNA Thermal Cycler (Perkin Elmer, Montréal, Québec, Canada), restriction enzymes and T4-DNA ligase were purchased from New England Biolabs (Mississauga, Ontario, Canada) and Gibco Life Technologies (Burlington, Ontario, Canada). DNA sequencing was provided by University of Ottawa Biotechnology Centre (Ottawa, Ontario, Canada) and Bio S&T (Montréal, Québec, Canada). RhoA and Cdc42 antibodies were purchased from Santa CruzBio Technology Inc. (Santa Cruz, California). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (Mississauga, Ontario, Canada). All protease inhibitors were purchased from Sigma (Oakville, Ontario, Canada).

Preparation of cDNAs encoding new C3-like chimeric proteins — cDNA encoding C3-transferase in the plasmid pGEX-2T was obtained from Natalie Lamarche (Department of Anatomy and Cell Biology, McGill University). To add additional sequences to the 3’ end, the stop codon was replaced with an EcoRI restriction site by polymerase chain
reaction (PCR) using the primers: 5’GAATTCTTTAGGATTGATAGCTGTGCC 3’ and 5’GGTGCGGCCCATCCTCCAAAA 3’. The PCR, product was cloned into an EcoRV restriction site in the pSTBlue-1 vector (Novagen, Madison, WI). The C3 PCR product was then sub-cloned into pGEX-4T (Amersham Pharmacia, Québec, Canada) using BamHI and EcoRI restriction sites, creating pGEX-4T/C3.

The shorter of the two Tat constructs, corresponding to aa 48-60, was made by annealing the oligonucleotides sequences 5’AATTCTATGGTCGTAAAAAACGTCGTCAACGTCGTCGTG 3’ and 5’GATACCAGCATTTTTTGCAGCAGTTGCAGCAGCACAGCT 3’. The annealed sequence was ligated into pGEX-4T/C3 at EcoRI and SalI restriction sites. A longer Tat construct (C3-03), corresponding to aa 27 to 72, was made by PCR. The template was plasmid SVCMV-Tat, a generous gift from Dr. Eric Cohen (Department of Microbiology and Immunology, Université de Montréal) and 5’GAATCCAAGCATCCAGGAAGTCAG 3’ and 5’TCAGTTCTCCTTCGCCCTCAGGCAGTCGTCGTG 3’ were used as primers. The PCR product was cloned into an EcoRV restriction site in pSTBlue-1 and further sub-cloned into pGEX-4T/C3, using the EcoRI restriction sites. C3-04 was constructed with oligonucleotide sequences corresponding to the third helix of Antennapedia; 5’AATTCCGCCCAGA TCAAGATTGGTTCCAGAATCGTGCAGTAAGTGGAAGAAGG 3’ and 5’GGCGGTCTAGTTCTAAACCAAGCTCTTAGCAGCGTAGTCACCTTCTTCCAGC 3’. These oligonucleotides were annealed and ligated into pGEX-4T/C3 at the EcoRI and SalI restriction sites. A vector containing the full length Antennapedia sequence cloned into pET-3a (pAHI), was a generous gift from Alain Prochiantz (Ecole Normal Supérieure, Paris, France). This vector was used to isolate, by
PCR, a 60 amino acid region encoding the full-length homeodomain, using primers:

5’ GGAATCCCGCAAACGCGCAAGGCAG 3’ and 5’ TCAGTTCTCCTTCTTT

CCACTTCATGCG 3’. The PCR product was cloned into pSTBlue-1 and subcloned into pGEX-4T/C3 using EcoRI, creating C3-05. Sequencing of this construct revealed a deletion mutation that altered the primary amino acid sequence, giving a proline-rich sequence resembling fusogenic peptides (23), and thus it was kept and tested. C3-06 was constructed by oligonucleotide sequences that coded for a highly basic and arginine-rich peptide corresponding to the reverse Tat sequence, 5’ AATTCAGAAGGAAAGAAAGAAGAAAAGAAGACTGCAGGC 3’ and 5’ GGCGGCCTGCAGTCTTCTTTTTCTTCTG 3’. These oligonucleotide sequences were annealed and ligated into pGEX-4T/C3, at EcoRI and NotI restriction sites. Plasmids were transformed into XL-1 blue competent cells except C3-06, in which DH5α competent cells were used. Plasmids were sequenced through the fusion region to the end of the peptide.

**Preparation of Recombinant Proteins** — Recombinant C3 and C3-like chimeric proteins were purified by affinity chromatography. Bacteria were grown in L-broth (10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L NaCl) with 50 µg/ml ampicillin (Roche, Québec, Canada), in a shaking incubator for 1.5 hrs at 37°C and 300 rpm. Isopropyl β-dithiogalactopyranoside (IPTG), (Gibco, Burlington, Ontario, Canada) was added to a final concentration of 0.5 mM, to induce the production of recombinant protein, and then the cultures were incubated for another 6 hours at 37°C and 250 rpm. The bacteria were pelleted by centrifugation in a GSA rotor (Sorval, Superspeed Centrifuge) at 7000 rpm for 6 minutes at 4°C. Each pellet was re-suspended in 10 ml of buffer A (50 mM Tris,
pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT plus PMSF (1 mM). All re-suspended pellets were pooled and transferred to a 100 ml plastic beaker on ice. The bacterial suspension was sonicated for 6 x 20 seconds, on ice, using a Branson Sonifier 450 probe sonicator (VWR, Québec, Canada). The lysate was centrifuged twice in a Sorvall SS-34 rotor at 16,000 rpm for 12 minutes at 4°C to clarify the supernatant. Glutathione-agrose beads (Sigma, Oakville, Ontario, Canada) were added to the cleared lysate and the preparation was placed on a rotator for 2-3 hours. The beads were washed 4 times with Buffer B, (Buffer A + 100 mM NaCl) and 2 times with Buffer C (Buffer B + 2.5mM CaCl$_2$). The final wash was removed until a thick slurry was created, and 20 U of thrombin (Calbiochem, San Diego, California) was added and the beads were shaken overnight at 4°C. The beads were loaded into an empty 20 ml column and 1 ml aliquots were collected after elution with PBS. The fractions containing the protein peak were pooled. To remove the thrombin form the protein sample, 100 µl of p-aminobenzamidine agarose beads (Sigma, Oakville, Ontario, Canada) were added and left mixing for 45 minutes at 4°C. The protein was centrifuged to remove the beads and then concentrated using a centriprep-10 concentrator (Millipore, Ontario, Canada). The concentrated protein was desalted by PD-10 column containing Sephadex G-25M (Amersham Pharmacia, Québec, Canada) and 10, 0.5 ml aliquots were collected. The appropriate aliquots were pooled, sterilized by filtration, and stored at – 80°C. Concentration of proteins was determined by protein assay (DC assay, Bio-Rad, Mississauga, Ontario, Canada). Purity of the sample was determined by SDS-PAGE, and confirmed by western blot with anti-C3 antibody (eg. Fig.1).
Cell Culture - PC-12 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), and differentiated by addition of 50 ng/ml of nerve growth factor (NGF). One day prior to use, 8 well chamber Lab-Tek slides (Nunc, Naperville, IL) were prepared by incubating 150 µl of poly-l-lysine (0.025 µg) (Sigma, Oakville, Ontario, Canada) for 4 hours and then drying 8g of myelin overnight in the laminar flow hood. To detach cells for plating, 2 ml of trypsin-EDTA (0.05%) was added and approximately 7500 cells were plated on the coated chamber slides. The cells were left at 37°C for 3-4 hours to allow them to settle. After the cells had adhered to the test substrate, the media was aspirated and fresh DMEM with 1% FBS, 1% P/S, 50 ng/ml of NGF, together with the appropriate amount of the test C3-like chimeric proteins were added. After 24 hours, the cells were fixed with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer. For control experiments with unmodified C3, NGF primed PC-12 cells were trypsinized, washed with scrape loading buffer (114 mM KCL, 15 mM NaCl, 5.5 mM MgCl2, and 10 mM Tris-HCl) and scraped with a rubber policeman in the presence, or absence of C3. For all experiments, at least four separate experiments were performed in duplicate.

Since myelin is phase dense, cells plated on myelin substrates were immunostained with β-III tubulin antibody (Sigma, Oakville, Ontario, Canada, 1:500), and detected with mouse HRP secondary antibody and revealed by 3,3’-diaminobenzidine. For each well, twelve images were collected with a 20X objective using a Zeiss Axiovert microscope (Oberkochen, Germany). The percent of cells with neurites of at least one cell body diameter in length were counted, and the longest neurite per cell was measured using Northern Eclipse Data analysis (Empix Imaging, Mississauga, Ontario, Canada) and
statistical unpaired student-t tests were completed with Microsoft Excel (Microsoft) and Sigmaplot.

NIH 3T3 and CHO cells were grown in DMEM with 10% FBS and 1% P/S. To detach the cells for plating 2ml of trypsin-EDTA (0.05%) was added and approximately 5000 cells were plated on the 8 well chamber slides. The cells were left at 37°C for 3-4 hours to allow them to settle. After the cells had adhered to the slides, the media was aspirated and fresh DMEM with 10% FBS, 1% P/S, together with varying concentrations of C3-05 was added. In experiments where Rho was activated by the addition of LPA, the cells were plated on 8 well chamber slides at a concentration of approximately 5000 cells per well, and then serum starved for 24 hours prior to treatment. The cells were then stimulated with LPA (100nM) for 15 minutes (24). The media was then aspirated and fresh DMEM with 1% P/S, together with 0.0025 µg/ml of C3-05 was added. After 24 hours the cells were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, and stained with phalloidin-TRITC (Sigma, Oakville, Ontario, Canada), phalloidin-rhodamin (Molecular Probes, Eugene, OR), or probed with an anti-vinculin antibody, 1:400, (Sigma, Oakville, Ontario, Canada). For all treatments at least three separate experiments were performed in duplicate. A minimum of six images per well, showing approximately 20 cells, was collected for detailed analysis. The micrographs shown in Figs 6 and 7A-B show individual cells representative of the total cell population.

To culture retinal ganglion neurons (RGCs), retinas were removed from postnatal day zero (P0) rat pups, and the cells were dissociated with 12.5 U/ml Papain in Hanks balanced serum solution (HBSS), 0.2 mg/ml DL-cysteine and 20 µg/ml bovine serum albumin (BSA). The dissociated cells were washed, added to DMEM with 10% FBS, 1%
P/S, 50 µg/ml BDNF and plated on inhibitory substrates, myelin (4 µg) or CSPG, in the presence or absence of C3-like chimeric proteins for 24 hours. The CSPG substrate (Chemicon, Temecula, California) was plated at a concentration of 0.001 µg in PBS and incubated at 37ºC overnight.

**Immunocytochemistry** — A polyclonal anti-C3 antibody was made by injecting a rabbit subcutaneously with 0.5 mg of C3 protein in Freund’s complete adjuvant and the rabbit was boosted four times in incomplete Freund’s adjuvant with 0.5 mg C3. Blood samples were tested until a high antibody titer was reached and then the serum was collected, purified and verified by ELISA (Sheldon Biotechnology Centre, McGill University). The specificity of the antibody was tested by western blot (Fig. 1). PC-12 cells were grown and plated on myelin-coated slides as described above. The cells were treated with 10 µg/ml of C3-05, or C3 added directly to the media and incubated at 37ºC for 24 hours. The cells were fixed with 4% PFA and probed with an anti-C3 antibody followed by FITC staining.

**Western Blots** — PC-12 cells treated for 24 hours with 25 µg/ml of C3, by scrape-loading, or 10 µg/ml of C3-05, added directly to the media, were washed twice with cold TBS, and lysed in 20mM Tris pH 8, 125 mM NaCl, 10% glycerol, 1% NP40, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin. CHO and NIH 3T3 cells were treated with 10 µg/ml of C3-05 and cell lysates were prepared as described above. Lysates were clarified by centrifugation and 10 µg of protein was separated on 12% acrylamide gels. After transfer to nitrocellulose, the membranes were either blocked with TBS containing 0.1% Tween 20 (TBS-T) and 3% BSA and incubated in blocking buffer with an anti-RhoA antibody (1:1000) (Santa Cruz, Santa Cruz, California), or blocked
with 5% powered milk and incubated in blocking buffer with an anti-C3 antibody (1:4000). The signals were revealed by an HRP-based chemiluminescent reaction (Pierce, Rockford, IL). Membranes probed with anti-RhoA antibody were stripped and re-probed with an anti-Cdc42 antibody (1:1000) (Santa Cruz, Santa Cruz, California).

Pull down assays to detect Rho-GTP — The activity assays were performed as previously described (25,26). PC-12 cells were grown on poly-l-lysine, or myelin coated 6 well culture dishes. After the cells settled (3-6 hours at 37°C), the media was aspirated and fresh media containing the test C3-like chimeric proteins was added to the cultures. NIH 3T3 cells were plated in 6 well culture dishes and incubated at 37°C for 3-6 hours. After the cells settled, the media was aspirated and fresh media containing the test C3-like chimeric proteins was added to the cultures. At indicated times (Fig. 8B), the cells were washed with ice cold Tris buffered saline (TBS) and lysed in RIPA buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF. Cell lysates were clarified by centrifugation at 13,000 g for 10 minutes at 4°C and incubated for 50 minutes at 4°C with GST- Rho binding domain (RBD) (a generous gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute) coupled to glutathione-agarose beads (Sigma, Oakville, Ontario, Canada). The beads were washed 4 times with cold Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.1 mM PMSF and eluted in sample buffer containing 40 mM DTT. Bound Rho-GTP eluted from the beads and total Rho from the original cell lysate was detected by western blot analysis using a monoclonal anti RhoA antibody (1:1000 in TBS-T, Santa Cruz). The quantification of Rho inactivation by C3-05 was by
densitometry using IQ MAC 1.2 software (Molecular Dynamics, Sunnyvale, Ca).

Densitometry values for untreated cells plated on myelin were normalized to correspond to 100% Rho activation. C3-05 treated cells were calculated as the percent inactivation compared to the normalized values.

RESULTS

We chose to test a number of different strategies to design C3-like chimeric proteins that could cross the plasma membrane by receptor independent mechanisms. Five C3-like chimeric proteins were constructed by adding DNA sequences encoding known membrane translocating peptides derived from Tat (C3-02, C3-03), Antennapedia (C3-04), a proline-rich fusogenic peptide (C3-05) and a basic, arginine-rich peptide (C3-06) to the 3’ end of the C3 cDNA. All five cDNAs encoding the C3-like chimeric proteins were expressed as GST fusion proteins in E.coli, purified, and their molecular weights verified by SDS-PAGE gel (eg. Fig. 1A). To confirm the presence of C3 in all five constructs, western blots using a polyclonal antibody raised against C3 were completed (eg. Fig.1B).

PC-12 cells typically extend neurites in response to NGF, but when plated on inhibitory substrates, this outgrowth is inhibited and the cells remain round (7). The ability to inactivate Rho and promote neurite outgrowth on inhibitory substrates was used as a bioassay to test the effectiveness of the new C3-like chimeric proteins. First, we examined the dose-response profile of unmodified C3. In previous experiments to inactivate Rho, we determined that scrape-loading was necessary to treat PC-12 cells with C3 (7). We found that even with the scrape-loading technique, high concentrations
of C3 were required to stimulate neurite outgrowth on myelin (Fig. 2). When C3 was added to the culture medium of pre-plated cells, C3 had no significant effect (Fig. 2).

To test the ability of the new C3-like chimeric proteins to promote neurite outgrowth, we performed dose response experiments with PC-12 cells plated on myelin substrates (Fig. 3). In these experiments, the C3-like chimeric proteins were added directly to the culture medium after pre-plating cells. This procedure was carefully performed to avoid any mechanical disruption of the cells. To establish the effective concentration ranges, preliminary experiments included a test concentration of 0.00025 µg/ml were completed, but none of the C3-like chimeric proteins were effective at this dose. Surprisingly, concentrations of 0.0025 µg/ml of C3-03, C3-05, and C3-06 lead to significant increases in both the number of cells extending neurites and the length of neurites compared to cells plated on myelin without treatment (Fig. 3A-B). This effective dose is 10,000-fold lower than that required with unmodified C3 using scrape-loading techniques (Fig. 2). At the highest concentrations tested, C3-03 was not effective, perhaps because of toxicity associated with the Tat sequence (14). C3-02 and C3-04 promoted significant neurite outgrowth at concentrations of 0.25 µg/ml and 2.5 µg/ml respectively and an increase in neurite length was observed at concentrations between the range of 0.025 µg/ml and 2.5 µg/ml (Fig.3A-B). At all concentrations, C3-05 and C3-06 stimulated neurite outgrowth on myelin substrates, with C3-05 giving the best results. These results indicated that all of the new C3-like proteins had some capacity to penetrate cells, inactivate Rho, and promote neurite outgrowth on inhibitory substrates. As C3-05 gave the best results, it was used for further testing.
ADP-ribosylation of Rho causes it to migrate with a larger apparent molecular weight on SDS gels (7,27). To study the ability of C3-05 to ADP-ribosylate Rho, we examined the electrophoretic mobility of RhoA by Western blot of cell lysates treated for 24 hours with 25 µg/ml of scrape-loaded C3, or 10 µg/ml of C3-05 added directly to the media (Fig. 4, top). Both scrape loaded C3 and C3-05 caused a similar molecular weight shift, confirming the ability of C3-05 to ADP-ribosylate Rho (Fig. 4, top). As a control for the specificity of this molecular weight shift effect, we stripped and re-probed the same blots for another member of the Rho GTPase family, Cdc42 (Fig. 4, bottom). Cdc42 did not show any change in mobility after treatment with C3-05, demonstrating that C3-05 maintains the same ADP-ribosylation specificity as unmodified C3.

We compared by immunocytochemistry the ability of C3 and C3-05 to enter cells and ADP-ribosylate Rho. PC-12 cells were plated on myelin substrates and treated with C3, C3-05, or left untreated. The cells were washed twice in cold TBS before preparing cell lysates. The proteins were separated on 12 % polyacrylamide gels, transferred to nitrocellulose and probed with a polyclonal anti-C3 antibody. A 27-kDa band was observed in the lysates of PC-12 cells treated with C3-05 (Fig. 5A). No band was detected in PC-12 cells treated with unmodified C3, indicating that no detectable amount of unmodified C3 was able to penetrate PC-12 cells without scrape loading. CHO and NIH 3T3 cells were incubated with 10 µg/ml of C3-05 for 24 hours, or left untreated. C3-05 was clearly detected in the cell lysates of both fibroblast cell lines tested (Fig. 5B). By immunocytochemistry of PC-12 cells plated on myelin, we observed no intracellular staining with unmodified C3, but staining was visible when cells were treated with C3-05 (Fig. 5B-C). These results further confirm the permeability of C3-05.
The activation of Rho in fibroblasts stimulates the assembly of actin stress fibers and focal adhesions. Microinjection of C3 into fibroblast cells results in the loss of stress fibers (1). To test if C3-05 is effective in crossing the cell membrane and inactivating Rho in fibroblasts, we treated CHO cells and NIH 3T3 cells with varying concentrations of C3-05, and stained the cells with phalloidin to visualize actin stress fibers (Fig. 6). When CHO and NIH 3T3 cells were plated in serum containing medium in the absence of C3-05, well-formed actin stress fibers were present (Fig. 6). In both cell lines the addition of C3-05 at concentrations as low as 0.0025 µg/ml dramatically reduced actin stress fiber formation. At concentrations of 0.025 µg/ml, or 0.25 µg/ml disassembly of actin stress fibers were almost complete. At 25 µg/ml, the highest concentration tested, CHO cells treated with C3-05 showed an altered morphology (Fig. 6).

The serum component lysophosphatidic acid (LPA) is a strong activator of Rho (25). Serum starved fibroblasts have very few visible stress fibers, however, the stimulation of such cells by LPA produces an intense network of actin stress fibers (1). To test if C3-05 is effective in reversing Rho activation by extracellular stimuli in fibroblasts, we stimulated serum starved CHO and NIH 3T3 cells with LPA (100nM) and then treated them with 0.0025 µg/ml of C3-05. The cells were stained with phalloidin to visualize the formation of actin stress fibers (Fig. 7A). In both cell lines, serum starved cells in the absence of LPA displayed a limited number of stress fibers. In contrast, when Rho was activated by LPA stimulation a dramatic increase in the formation of stress fibers occurred. The treatment of LPA stimulated cells with 0.0025 µg/ml of C3-05 resulted in a substantial decrease in the formation of actin stress fibers visualized by phalloidin stain.
(Fig. 7A). Therefore C3-05 can reverse the formation of actin stress fibers induced by LPA.

Focal adhesions are sites of attachment between cells and their extracellular matrices that are regulated by Rho (1). To examine the effects of C3-05 on focal adhesions, we stimulated serum starved NIH 3T3 cells with 100 nM LPA, treated them with 0.0025 µg/ml of C3-05 and visualized focal adhesion complexes with an anti-vinculin antibody (Fig. 7B). Serum starvation of NIH 3T3 cells prevented formation of focal adhesions. LPA stimulation to activate Rho promoted an increased formation of focal adhesions compared to the serum starved controls (Fig. 7B). Treatment of the LPA stimulated cells with 0.0025 µg/ml C3-05 prevented the LPA-induced augmentation of focal contacts (Fig. 7B).

Rho activity can be measured using GTP-bound Rho pull down assays. When cells are plated on myelin substrates, an increase in the amount of active Rho was observed compared to cells plated on poly-l-lysine (Fig. 8A). When PC-12 cells were plated on myelin and treated with 0.0025 µg/ml, 0.025 µg/ml, and 0.25 µg/ml of C3-05, there was a dramatic reduction in the levels of active Rho. Treatment with C3-05 decreased the amount of active Rho below levels observed from PC-12 cells plated on growth promoting poly-l-lysine substrates (Fig. 8A). To determine the duration of this C3-05 induced Rho inactivation, PC-12 cells plated on growth inhibitory substrates were incubated with C3-05 and lysed at different time points after the addition of C3-05. Rho activation was completely reversed at 0.025 µg/ml and 0.25 µg/ml after 24 hours. At all concentrations tested, the ability of C3-05 to inactive Rho peaked at 24 hours. Rho inactivation began to diminish after 36 hours with the complete activation of Rho
regained in cells treated with 0.0025 µg/ml at 48 hours (Fig. 8B). Cells treated with all three concentrations maintained their differentiation state for at least 48 hours after treatment (data not shown). These results show that myelin causes Rho activation resulting in inhibition of neurite outgrowth, and that this inhibition can be completely reversed by treatment with C3-05.

In NIH 3T3 cells Rho activation is correlated with the formation of actin stress fibers (Fig. 4). Pull down assays of homogenates prepared from NIH 3T3 cells grown in the presence of serum showed high Rho activation levels, in agreement with the spread morphology of these cells (Fig. 8C). Treatment with varying concentrations of C3-05 at 0.0025 µg/ml, 0.025 µg/ml and 0.25 µg/ml for 24 hours decreased Rho activation (Fig. 8C-D). These results confirm that C3-05 inactivated Rho in fibroblasts.

To further support the ability of C3-like chimeric proteins to promote neurite outgrowth on inhibitory substrates, we examined the response of primary cultures plated on inhibitory substrates to C3-05 treatment. Purified retinal ganglion cells (RGCs) were plated on myelin, or CSPG substrates and treated with varying concentrations of C3-05 for 24 hours. During the RGC dissection great care was taken in order to try to limit the amount of mechanical manipulation of the cells, however, the isolation protocol requires that some triturating take place in order to dissociate and separate the cells. When RGCs are plated on inhibitory substrates, they maintained a similar round appearance to PC-12 cells plated on myelin. Treatment of RGCs with C3-05 promoted neurite outgrowth and increased neurite length on both myelin and CSPG substrates (Fig. 9A-F). In contrast to the wide range of concentrations shown to be effective in experiments with PC-12 cells, a narrower range of C3-05, 0.025 µg/ml to 50 µg/ml, promoted neurite outgrowth and
increased neurite length on myelin (Fig. 9A-D). In the case of RGCs plated on CSPG effective concentration ranges of 0.0025 µg/ml to 50 µg/ml were observed (Fig. 9A,B, E,F).

DISCUSSION

Here we report the construction of five new C3-like chimeric proteins, all of which possess the ability to translocate across the plasma membrane to ADP-ribosylate and inactivate Rho. By a bioassay in which PC-12 were cells plated on growth inhibitory myelin substrates, we have shown that, to varying extents, all five C3-like chimeric proteins promoted neurite outgrowth. Based on our experiments with unmodified C3, which must be scrape loaded, we suggest that the ability of these new C3-like proteins to promote neurite outgrowth at such low concentrations is due to their increased cellular permeability. The differences observed in promoting neurite outgrowth between the five C3-like chimeric proteins might result from the different methods used by the various transmembrane carrier peptides to enter cells.

We tested two different transport sequences derived from the Tat protein of the human immunodeficiency virus (HIV). This protein has been reported to enter cells, carry protein cargo into cells, and even cross blood brain barrier (13). The mechanism whereby Tat transports cargo across the plasma membrane is still not completely understood. Tat internalization is not decreased at 4 °C, or in the presence of endocytosis inhibitors (14). There is uncertainty, however, whether its uptake is receptor mediated because Tat binds to specific cell membrane proteins (28). We found that C3-03, the longer Tat peptide sequence, was more efficient at promoting neurite outgrowth than C3-02, the shorter Tat peptide. However, the longer sequence may have some cellular
toxicity (14), a finding consistent with the decreased ability to promote neurite growth at high concentrations (Fig. 3).

The third helix of the Antennapedia homeodomain can cross cell membranes by both energy and receptor independent mechanisms (16). Antp is a basic peptide that interacts with the charged phospholipids on the outer side of the cell membrane, causing destabilization of the lipid bilayer and the formation of inverted micelles. The formation of this hydrophobic structure allows the Antp and protein cargo to travel freely across the membrane, releasing the transported protein inside the cell once the hydrophobic pocket opens (16,17,29). One drawback to this family of transport peptides is that they lose their translocating ability when they bind to double stranded DNA (30). We found that C3-04 containing the Antp sequence was an effective carrier, but only within a narrow concentration range (Fig. 3).

Proline-rich peptides can also act as receptor independent delivery peptides. Fusogenic peptides contain both hydrophobic and hydrophilic amino acids, which form amphiphilic α-helical structures. A critical component of these proteins are proline residues (19,23). Studies where site-directed mutation changing single proline residues of the PH-30α fusogenic protein, active in sperm-egg fusion, shows that prolines are critical for the fusogenic activity (23,31). The membrane translocating sequence (MTS) of Kaposi fibroblast growth factor, a known transport peptide, contains 3 proline residues spaced 5 to 7 amino acids apart (21). The spacing of prolines in this MTS peptide are similar to that in C3-05, which also possesses 3 prolines spaced 6 to 8 amino acids apart. Furthermore, these proline residues may explain why C3-05 was the most effective C3-like chimeric protein tested. When proline residues were added to Antp translocating
sequences, Antp and its cargo were only present in the cytoplasm and not in the nucleus (32). The possibility that proline residues may restrict the membrane translocating peptides and their cargo to the cytoplasm would increase the ability of C3-05 to inactive Rho, a cytoplasmic protein.

Highly basic, arginine-rich peptides are another class of transport peptides. A simple string of seven, or more arginines covalently linked to a fluorescein moiety at the N-terminal was able to cross the cell membrane when analyzed by flow cytometry (21). These transport peptides were more effective than both Tat and Antennapedia when compared directly (20). C3-06 contains a basic transport sequence, which corresponding to the reverse Tat sequence. As previously reported (20), we also found it to be a more effective transport sequence than either Tat, or Antennapedia. C3-06 contains three arginine residues at the amino end of the peptide end, compared to the Tat translocation peptide used in C3-02, which contains one arginine, and two lysine residues at the amino terminal. The increased ability of C3-06 to penetrate PC-12 cells and promote neurite outgrowth suggests that N-terminal residues are important for cellular uptake. Furthermore, arginine residues are more effective then lysine residues in inducing cellular uptake (20).

Neurite outgrowth and neurite length profiles of PC-12 cells treated with C3-02 and to a lesser degree C3-03 and C3-04 had a normal distribution, (Fig.3) showing increased neurite outgrowth when cells were treated with low to moderate concentrations, but not with high concentrations. High concentrations of C3-02, C3-03 and C3-04 may have toxic effects on PC-12 cells. Previously, two other permeable C3 fusion proteins have been produced, one with Diphtheria-toxin B subunit, called DC3B (9) and another
that is a C2 toxin-C3 fusion protein, called C2IN-C3 (10). Our three most effective C3-like chimeric proteins, C3-03, C3-05, and C3-06 all worked at a much lower dose than DC3B (0.6 µg/ml) (9) and C2IN-C2 (0.2-0.3 µg/ml) (10), being effective at 0.0025 µg/ml. The lower effective dose of our C3-like proteins might be because they enter cells by receptor-independent mechanisms, and therefore should not be trapped within endocytotic compartments. In addition, C2IN-C3 is not independently cell permeable because the C2II, the binding component of C2 toxin, must be present to induce the uptake of the C2IN-C3 fusion protein by endocytosis. Furthermore, C2IN is a relatively large peptide consisting of 225 amino acids, which when attached to C3-transferase nearly doubles its molecular weight. All of the transport sequences used in this paper are under 50 amino acids, which may enhance uptake, as the total size of the protein is not dramatically increased.

When neuronal cells are plated on myelin they become round and do not grow neurites (7). Previously, we have suggested that myelin-derived growth inhibitory proteins directly activate Rho (33). Here we demonstrate the first evidence that inhibitory substrates activate Rho. GTP-bound Rho assays showed that myelin alone activated Rho when compared to cells plated on poly-l-lysine substrates (Fig. 7). Cells plated on myelin showed a 4 to 5-fold increase in cellular active Rho compared to cells plated on poly-l-lysine (Fig. 7). Treatment with C3-like chimeric proteins not only reversed the myelin induced Rho activation, but sustained this decrease for 36 to 48 hours. For all 3 concentrations tested, peak Rho inactivation appeared 24 hours after treatment, and then began to decrease. Possibly, the decrease occurred because the C3-like chimeric proteins...
were no longer active, or had all been taken up. We did not test whether the addition of more C3-05 to the culture media could sustain Rho inactivation for longer periods.

In previous *in vivo* experiments using unmodified C3, a robust period of regeneration of retinal ganglion cells in the optic nerve was observed after treatment (7). Future studies will address the ability of these new C3-like proteins to help axon regeneration and repair after CNS injury. These C3-like chimeric proteins, therefore, may improve the extent of regeneration in the central nervous system following spinal cord injury.

The pathological progression of cancer involves abnormal cell growth, resulting in the formation of tumors, and increased cell motility, causing invasive properties and metastasis. Recent studies provide evidence that Rho A, B and C, all substrates for C3, play a role in both tumor development and metastatic progression by regulating the growth and motility of cells (34-40). For example, over expression of Rho is implicated in tumor formation of neck squamous cell cancer, aggressive ductal adenocarcinoma of the pancreas and inflammatory breast cancer (34,37,38). In culture, fibroblasts transfected with active Rho develop alterations in morphology and grow at higher densities than untransfected cells (35,39). The regulation of cell proliferation by active Rho in fibroblasts is inhibited by C3 treatment. This inhibition of cell proliferation is evident one day after treatment with C3, correlating with the ADP-ribosylation of Rho (41). Other studies suggest Rho plays a role in tumor metastasis. In contrast to neuronal cells, where Rho activation inhibits cell motility (Fig. 3C), Rho activation in tumor cells can increases invasiveness and motility of cells. Experiments with metastatic tumors induced in mice show that the RhoC gene is over expressed compared to the expression
profiles of other genes as determined by DNA array analysis (40). The transfection of dominant negative Rho in highly metastatic human tumor cells suppresses the number of metastatic tumors observed in mice (40). The C3-like chimeric proteins described here may prove useful therapeutically as several experiments have demonstrated that the inactivation of Rho has a role in limiting abnormal cell growth and metastasis (40-42).

The characterization of five C3-like chimeric proteins demonstrates biochemical properties similar to those seen in previous experiments using C3 (7). Addition of the translocating peptide sequences to the carboxyl terminal of C3 does not interfere with its ability to ADP-ribosylate Rho and cause neurite outgrowth on inhibitory substrates in neuronal cells, or the reduction of actin stress fibers in fibroblasts. The transport sequences do not affect the activity of C3, as C3-like chimeric proteins were still active in the lysates of cells, determined by pull down assay, several days after treatment. By increasing the efficiency of C3 entry into cells, smaller volumes and lower concentrations of C3 can be used without disruptive methods, resulting in possible therapeutic roles in the regulation of tumor formation in cancers and the regeneration of neurons after traumatic injury.
REFERENCES

1. Ridley, A. J., and Hall, A. (1992) *Cell* **70**, 389-99.
2. Hall, A. (1998) *Science* **279**, 509-14.
3. Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994) *J Cell Biol* **126**, 801-10.
4. Tsigi, G., Fischer, D. J., Sebok, A., Yang, C., Dyer, D. L., and Miledi, R. (1996) *J Neurochem* **66**, 537-48.
5. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) *Mol Cell Biol* **17**, 1201-11.
6. Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W. D., and McKerracher, L. (2002) *Journal of Neuroscience*. In Press.
7. Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tsigi, G., and McKerracher, L. (1999) *J Neurosci* **19**, 7537-47.
8. Jin, Z., and Strittmatter, S. M. (1997) *J Neurosci* **17**, 6256-63.
9. Boquet, P., Popoff, M. R., Gir, M., Lemichez, E., and Bergez-Aullo, P. (1995) *Methods Enzymol* **256**, 297-306.
10. Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1998) *Infect Immun* **66**, 1364-9.
11. Ohishi, I., and Yanagimoto, A. (1992) *Infect Immun* **60**, 4648-55.
12. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994) *Proc Natl Acad Sci U S A* **91**, 664-8.
13. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *Science* **285**, 1569-72.
14. Vives, E., Brodin, P., and Lebleu, B. (1997) *J Biol Chem* **272**, 16010-7.
15. Bloch-Gallego, E., Le Roux, I., Joliot, A. H., Volovitch, M., Henderson, C. E., and Prochiantz, A. (1993) *J Cell Biol* **120**, 485-92.
16. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J Biol Chem* **269**, 10444-50.
17. Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., and Prochiantz, A. (1996) *J Biol Chem* **271**, 18188-93.
18. Deber, C. M., Brandl, C. J., Deber, R. B., Hsu, L. C., and Young, X. K. (1986) *Arch Biochem Biophys* **251**, 68-76.
19. Du, C., Yao, S., Rojas, M., and Lin, Y. Z. (1998) *J Pept Res* **51**, 235-43.
20. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) *Proc Natl Acad Sci U S A* **97**, 13003-8.
21. Rojas, M., Donahue, J. P., Tan, Z., and Lin, Y. Z. (10998) *Nat Biotechnol* **16**, 370-5.
22. Morris, M. C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001) *Nat Biotechnol* **19**, 1173-6.
23. Niidome, T., Kimura, M., Chiba, T., Ohmori, N., Mihara, H., and Aoyagi, H. (1997) *J Pept Res* **49**, 563-9.
24. Tsigi, G., Fischer, D. J., Sebok, A., Marshall, F., Dyer, D. L., and Miledi, R. (1996) *J Neurochem* **66**, 549-58.
25. Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999) *Embo J* **18**, 578-85.
26. Ren, X. D., and Schwartz, M. A. (2000) *Methods Enzymol* **325**, 264-72.
27. Morii, N., and Narumiya, S. (1995) *Methods Enzymol* 256, 196-206.
28. Weeks, B. S., Desai, K., Loewenstein, P. M., Klotman, M. E., Klotman, P. E., Green, M., and Kleinman, H. K. (1993) *J Biol Chem* 268, 5279-84.
29. Berlose, J. P., Convert, O., Derossi, D., Brunissen, A., and Chassaing, G. (1996) *Eur J Biochem* 242, 372-86.
30. Derossi, D., Chassaing, G., and Prochiantz, A. (1998) *Trends Cell Biol* 8, 84-7.
31. Pecheur, E. I., Sainte-Marie, J., Bienvene, A., and Hoekstra, D. (1999) *J Membr Biol* 167, 1-17.
32. Prochiantz, A. (1999) *Ann N Y Acad Sci* 886, 172-9.
33. Ellezam, B., C., D., Winton, M. J., Loy, L., Dergham, P., Sellés-Navarro, I., and McKerracher, L. (2002) *Progress in Brain Research* 137.
34. Abraham, M. T., Kuriakose, M. A., Sacks, P. G., Yee, H., Chiriboga, L., Bearer, E. L., and Delacure, M. D. (2001) *Laryngoscope* 111, 1285-9.
35. del Peso, L., Hernandez-Alcoceba, R., Embade, N., Carnero, A., Esteve, P., Paje, C., and Lacal, J. C. (1997) *Oncogene* 15, 3047-57.
36. Prendergast, G. C., Khosravi-Far, R., Solski, P. A., Kurzawa, H., Lebowitz, P. F., and Der, C. J. (1995) *Oncogene* 10, 2289-96.
37. Suwa, H., Ohshio, G., Imamura, T., Watanabe, G., Arii, S., Imamura, M., Narumiya, S., Hiai, H., and Fukumoto, M. (1998) *Br J Cancer* 77, 147-52.
38. van Golen, K. L., Wu, Z. F., Qiao, X. T., Bao, L., and Merajver, S. D. (2000) *Neoplasia* 2, 418-25.
39. Perona, R., Esteve, P., Jimenez, B., Ballesteros, R. P., Ramon y Cajal, S., and Lacal, J. C. (1993) *Oncogene* 8, 1285-92.
40. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000) *Nature* 406, 532-5.
41. Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S., and Narumiya, S. (1993) *Oncogene* 8, 1449-55.
42. Imamura, F., Shinkai, K., Mukai, M., Yoshioka, K., Komagome, R., Iwasaki, T., and Akedo, H. (1996) *Int J Cancer* 65, 627-32.
Acknowledgments — We thank Charles Essagian and Marc Dufresne for their technical help. We are grateful to Dr. E Cohen (Université de Montréal) for providing the SVCMV-TAT vector and to Dr. A Prochiantz (Ecole Normal Supérieure) for providing the pAHI vector.

* This work was supported by the Canadian Institutes of Health Research (CHIR), the Natural Sciences and Engineering Research Council of Canada (NSERC), and BioAxone Thérapeutique, Inc.

1The abbreviations used are C3, C3-ADP-ribolytransferase; CSPG, chondroitin sulfate proteoglycan; PCR, polymerase chain reaction; Tat, human immunodeficiency virus transactivator protein; Antp, Antennapedia homeodomain; DTT, Dithiotheitol; PMSF, Phenylmethyl-sulfonyl fluoride; IPTG, isopropyltho-β-D-galactoside; PBS, phosphate buffered saline; TBS, Tris buffered saline; TBS-T, Tris buffered saline and 0.1% Tween-20; HIV, human immunodeficiency virus; DT, Diptheria toxin; DMEM, Dulbecco’s Modified Eagle Medium; NGF, nerve growth factor; BDNF, brain-derived growth factor; aa, amino acid; LPA, lysophosphatidic acid.
Fig. 1. **Purification of recombinant C3.** C3 was expressed as a GST fusion protein and purified from bacterial lysates by affinity chromatography. A, protein samples from the bacterial lysate (*LYSATE*) and the protein after purification and filter sterilization (*PURIFIED PROTEIN*). B, Western blot of samples of cell lysates (*LYSATE*) and purified protein (*PURIFIED PROTEIN*) probed with a C3 antibody.

Fig. 2. **C3 promotes neurite growth on inhibitory substrates only after scrape loading.** PC-12 cells plated on myelin substrates, were treated with C3 by scrape loading (*dark gray bars*), or by its addition directly into the culture media (*light gray bars*). Concentrations of C3 between 0.0025 µg/ml and 50 µg/ml were tested (*x-axis*). After 24 hours cells were fixed, stained and counted. A, the percentage of cells that extended neurites longer than 1 cell body diameter (*neurite outgrowth*); B, the length of the longest neurite per cell (*neurite length*). The average counts are shown for four or more experiments each performed in duplicate. Error bars represent the SEM, * is statistically significant (*p* ≤ 0.05).

Fig. 3. **C3-like chimeric proteins efficiently promote neurite outgrowth on myelin substrates.** PC-12 cells were plated on myelin substrates in the absence (*black bars*), or presence of C3-like proteins (*C3-02, white bars; C3-03, light gray bars; C3-04, gray bars; C3-05, dark gray bars; C3-06, charcoal bars*) at increasing concentrations, between 0.0025 µg/ml and 50 µg/ml, for 24 hours. A, the percent of cells that extended neurites (*neurite outgrowth*); B, the length of the longest neurite per cell (*neurite length*). Test proteins were added to the culture media of pre-plated cells. The data represents the
average of for four or more experiments performed in duplicate +/- the SEM. * indicates significance differences compared to untreated cells (p ≤ 0.05). C, micrographs showing PC-12 cells treated at the lowest concentration of the test C3-like proteins (0.0025 µg/ml).

Scale bar, 50 µm.

Fig. 4. ADP-ribosylation of Rho in PC-12 cells. PC-12 cells plated on myelin substrates, were untreated (CTRL), scrape loaded with 25 µg/ml unmodified C3 (C3-SCRAPE), or treated with 10 µg/ml C3-05 added directly to the media, and incubated for 24 hours. Top, western blot of cell lysates probed with an anti-RhoA antibody. ADP ribosylation causes a shift in the apparent molecular weight of RhoA (6). Bottom, the same membrane was stripped and re-probed with an anti-Cdc42 antibody.

Fig. 5. Transmembrane delivery of C3-05 into PC-12, CHO and NIH 3T3 cells. A, western blot of PC-12 cell lysates prepared from cells treated with 10 µg/ml of C3-05 (C3-05), 10 µg/ml of C3 (C3), and untreated cells (CTRL) and probed with a polyclonal anti-C3 antibody. B, western blots of CHO and NIH 3T3 cell lysates prepared from cells treated with 10 µg/ml of C3-05 (C3-05) and untreated cells (CTRL) and probed with a polyclonal C3 antibody. C, D, PC-12 cells plated on myelin substrates, were incubated with 10 µg/ml C3; or D, 10µg/ml C3-05 for 24 hours, and immunostained with a polyclonal anti-C3 antibody. Scale bar, 50 µm.
Fig. 6. **C3-05 reverses stress fiber formation in CHO and NIH 3T3 cells.**

Micrographs of phalloidin stained CHO and NIH 3T3 cells treated with increasing concentrations of C3-05 (0.0025 µg/ml to 25µg/ml) for 24 hours. The chimeric C3-like protein was added directly to the media of pre-plated cells. All experiments were performed in duplicate at least 3 separate times. Micrographs show individual cells representative of the total cell population observed. *Scale bar*, 50 µm.

Fig. 7. **C3-05 reverses LPA induces stress fibers and focal adhesions.** A. Serum starved CHO and NIH 3T3 cells were stimulated with 100 nM of LPA, treated with 0.0025 µg/ml C3-05 for 24 hours and stained with phalloidin to visualize stress fiber formation. B, NIH 3T3 cells were stimulated with 100 nM of LPA, treated with 0.0025 µg/ml of C3-05 and immunostained with an anti-vinculin antibody. Three experiments were performed in duplicate. Micrographs show individual cells representative of the total cell population observed. *Scale bar*, 50 µm.

Fig. 8. **Inactivation of Rho by C3-05.** A, assay for cellular GTP-bound Rho. PC-12 cells were plated on poly-l-lysine (*lane 1*), or myelin, (*lanes 2-5*) substrates with increasing concentrations, between 0.0025 µg/ml and 0.25 µg/ml, of C3-05 for 24 hours. The cells were washed, lysed, and GTP-bound Rho was isolated by pull-down assay. GTP-bound Rho (*top*) and total Rho (*bottom*) were detected by western blot with a Rho A antibody. B, measurement of Rho inactivation over time after treatment with C3-05 at different concentrations. C,GTP-bound Rho (*top*) and total Rho (*bottom*) of NIH 3T3 cells were
detected by western blot with an anti-RhoA antibody.  \( D \), measurement of Rho inactivation 24 after treatment of NIH 3T3 cells with C3-05 at different concentrations.

Fig. 9. **C3-05 promotes neurite outgrowth from retinal neurons plated on inhibitory myelin or CSPG substrates.** Retinal neurons plated on myelin substrates, (black bars); or CSPG substrates, (gray bars), were treated with increasing concentrations, between 0.0025 \( \mu \)g/ml and 50 \( \mu \)g/ml, of C3-05 for 24 hours. \( A \), the percentage of cells with neurites longer than 1 cell body diameter (neurite outgrowth); \( B \), the length of the longest neurite per cell (neurite length). The data represents the average of four or more experiments performed in duplicate +/- the SEM. * indicates significance differences compared to untreated cells \( (p \leq 0.05) \). \( C-F \), micrographs of retinal neurons plated on (C, E) myelin, or (E, F) CSPG substrates. \( C-D \), Controls without treatment do not grow neurites. \( D-F \), 2.5 \( \mu \)g/ml of C3-05 added to the culture media. *Scale bar*, 50 \( \mu \)m.
Figure 1
Figure 2
Figure 3

A

NEURITE OUTGROWTH (% ± SEM)

CONCENTRATION (µg/ml)

B

NEURITE LENGTH (µm ± SEM)

CONCENTRATION (µg/ml)

C

myelin

C3-02

C3-03

C3-04

C3-05

C3-06
Figure 4
Figure 5
Figure 6

| C3-05 (µg/ml) | 0 | 0.0025 | 0.025 | 0.25 | 25 |
|---------------|---|--------|-------|------|----|
| CHO          | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| NIH 3T3      | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |
Figure 7

A

| LPA (100nM) |
|-------------|
| C3-05 (µg/ml) |
| 0 | 0 | 0.0025 |

CHO

NIH 3T3

B

| LPA (100nM) |
|-------------|
| C3-05 (µg/ml) |
| 0 | 0 | 0.0025 |

NIH 3T3

Figure 7
Figure 8
Figure 9
**Figure Legend Placement**

Figure 1 – no preference
Figure 2 – underneath figures A and B
Figure 3 – to the right of figure B, underneath figure C
Figure 4 – no preference
Figure 5 – no preference
Figure 6 – no preference
Figure 7 – no preference
Figure 8 – no preference
Figure 9 – to the right of figure B, underneath figure C
Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates
Matthew J. Winton, Catherine I. Dubreuil, Dana Lasko, Nicole Leclerc and Lisa McKerracher

J. Biol. Chem. published online June 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201195200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts