Journal of Cell Science 107, 195-204 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994
JCS8400

An amino-terminal domain of the growth-associated protein GAP-43 mediates its effects on filopodial formation and cell spreading

Stephen M. Strittmatter*, Dario Valenzuela and Mark C. Fishman

Developmental Biology Laboratory, Massachusetts General Hospital and Departments of Neurology and Medicine, Harvard Medical School, Massachusetts General Hospital-East, 149 13th Street, Charlestown, MA 02129, USA

*Present address: Department of Neurology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT, USA

SUMMARY

GAP-43 is a neuronal protein that is believed to be important to neuronal growth and nerve terminal plasticity. It is enriched on the inner surface of growth cone membranes, a localization that may depend upon palmitoylation of Cys3 and Cys4. It is a major substrate for protein kinase C, which phosphorylates Ser41. Isolated GAP-43 can bind to actin and to calmodulin, and can activate the heterotrimeric GTP-binding proteins, G_o and G_i. A peptide consisting of the GAP-43 sequence 39-55 binds calmodulin, and an amino-terminal GAP-43 (1-10) peptide activates G_o, suggesting that these stretches may be functional domains of the intact protein.

When expressed in non-neuronal cells, GAP-43 enhances filopodial extension and has effects upon cell spreading. We have examined the effects of various GAP-43 domains upon this assay, by expression of GAP-43, GAP-43 mutant proteins, and GAP-43-CAT fusion proteins in COS-7 cells. We find that the amino terminus (Met-Leu-Cys-Cys-Met-Arg-Arg-Thr-Lys-Gln) is an important contributor to these effects on cell shape. A GAP-43 protein mutant in Cys3 and Cys4 does not bind to the membrane, and is inactive. Mutants in Arg6 or Lys9 also are inactive, although they remain localized to particulate fractions; Arg7 mutants are active. A chimeric gene consisting of GAP-43 (1-10) fused to chloramphenicol acetyl transferase (CAT) also causes cell shape changes. As for GAP-43, the effects of this fusion protein are abolished by mutations of Cys3, Cys4, Arg6 or Lys9, but not by mutation of Arg7. Therefore, the cell surface activity of transfected GAP-43 depends upon its amino terminus, although other domains may regulate it in this regard.

Since the amino-terminal domain includes the peptide stretch known to be capable of activating G_o and G_i, we examined the effect of GAP-43 on a G_i-regulated second messenger system, the inhibition of cAMP production in A431 cells. A431 cells stably transfected with GAP-43 spread less well than do controls. In addition, they evidence decreased levels of forskolin-stimulated cAMP, consistent with chronic stimulation of G_i. Stimulation of adenylate cyclase by isoproterenol reverses the GAP-43-induced changes in cell shape. This suggests that G protein stimulation is involved in GAP-43 effects upon cell shape.

Key words: neuronal growth cone, GAP-43 (neuromodulin), GTP-binding protein, signal transduction, cell spreading, axonal growth

INTRODUCTION

As a growth cone during development and regeneration, the nerve terminal is motile, extending and retracting filopodia and lamellipodia, while identifying appropriate pathways and targets (Lockerbie, 1987; Strittmatter and Fishman, 1991). The molecular basis of these alterations in neuronal structure is poorly understood. A central role in neuronal plasticity has been suggested for the protein GAP-43 because of its high concentration in the growth cone membrane and because of its heightened level of expression during axonal growth and regeneration, and in adult CNS regions where plasticity is prominent (reviewed by Benowitz and Routtenberg, 1987; Fishman, 1989; Skene, 1989; Strittmatter et al., 1992). That GAP-43 may enhance neurite extension has been suggested by the reduced neuritogenesis in the presence of intracellular anti-GAP-43 antibodies (Shea et al., 1991), and by the enhanced NGF responsiveness of PC-12 cells that overexpress GAP-43 (Yankner et al., 1990). However, PC-12 cells lacking the protein can still extend neurites (Baetge and Hammang, 1991), suggesting that while GAP-43 might alter a propensity to neurite growth, it is not itself essential. In other words, GAP-43 might enhance or diminish the effects of other growth regulators, without itself directly causing growth.

Support for this notion comes from observations with isolated proteins, which indicate an interaction of GAP-43 with the transduction machinery of the growth cone. The protein has been reported to alter inositol phosphate levels by modulating phosphatidylinositol-1-phosphate kinase activity (Oestreicher et al., 1983). GAP-43 can bind to calmodulin with an affinity of 1 to 3 μM at physiological ionic strengths (Alexander et al., 1987). The calmodulin binding domain has been localized to

the residues 39-55 of GAP-43 (Alexander et al., 1988; Chapman et al., 1991). Protein kinase C phosphorylates serine 41 of rat GAP-43 (Coggins and Zwiers, 1989) and this prevents calmodulin/GAP-43 interaction (Alexander et al., 1988; Chapman et al., 1991). GAP-43 may also alter cell shape by binding to the membrane skeleton (Meiri and Gordon-Weeks, 1990) and actin filaments (Strittmatter et al., 1992; Widmer and Caroni, 1993).

We have noted that GAP-43 increases GTP-binding to G_o by enhancing guanine nucleotide exchange (Strittmatter et al., 1990, 1991). N-terminal GAP-43(1-10) and GAP-43(1-25) peptides, but not GAP-43(1-6) peptide, stimulate G_o (Sudo et al., 1992). These findings are of particular interest because G_o, and to a lesser extent G_i, is highly concentrated in the growth cone membrane (Strittmatter et al., 1990; Edmonds et al., 1990). Presumably, they are coupled there to receptors for extracellular ligands. Certain neurotransmitters that act via G proteins do affect growth cone shape (Haydon et al., 1984; Lankford et al., 1988; Rodrigues and Dowling, 1990). The hypothesis that an intracellular protein might regulate G proteins suggests a new dimension for this transduction system, since, except for phosducin (Bauer et al., 1992), other previously described regulators of the G protein cascade are transmembrane receptors for extracellular ligands (Gilman, 1987; Ross, 1989).

To develop an assay for the cellular function of GAP-43, we chose to express this protein in cells that normally lack the molecule. Since growth cone activity appears to involve mechanisms conserved to some degree among all motile cells (Smith, 1988; Bray and White, 1988; Bray and Hollenbeck, 1988), we reasoned that GAP-43, even though expressed at highest levels in neurons, might be able to interact with the cell shape machinery of non-neuronal cells. GAP-43-expressing CHO and COS cells exhibit a transiently enhanced propensity to extend filopodia when trypsinized and plated (Zuber et al., 1989a). While this manuscript was in preparation, these observations were confirmed and extended by Widmer and Caroni (1993), who found that expression of GAP-43 increases filopodial formation and alters cell spreading in L6 and COS-7 cells. Cells expressing chick GAP-43 with a Ser42 point mutation that prevents phosphorylation spread less extensively than control cells, and cells expressing a phosphorylation-mimicking mutation spread to a greater extent than control cells.

We have employed this assay to define an active domain within the protein. We find that a short N-terminal, G-protein-activating segment is critical for activity in this morphological assay, and that specific residues in this region are essential.

MATERIALS AND METHODS

GAP-43 expression vectors and DNA transfection

The synthesis of many of the plasmids has been described previously (Zuber et al., 1989a,b). In brief, the GAP-43 plasmids were all derived from pCDM-8 and contain an SV40 origin of replication, and the rat GAP-43 cDNA sequence under the control of a CMV promotor. The point mutations of Arg6 to Gly, of Arg7 to Gly and of Lys9 to Gly were created from the GAP-43 vector by oligonucleotide-directed mutagenesis, as described previously for the Cys3,4 to Thr substitutions. The CAT and GAP-43/CAT expression vectors were constructed as described previously (Zuber et al., 1989a,b). The GAP-43(1-10)/CAT constructs encode a stretch of 17 amino acid residues, VDLQASLARFSGAKEAK, linking GAP-43 to CAT. The GAP-43(1-40)/CAT and GAP-43(1-6)CAT proteins are linked by ARVDLQASLARFSGAKEAK. All mutations and fusions were confirmed by DNA sequencing.

COS-7 cells were transfected with equal amounts of different DNAs by the DEAE-dextran method or by electroporation. Forty hours after transfection, cells were analyzed in the filopodial and spreading assays.

A431 epithelial cells were maintained in DMEM, 7.5% fetal bovine serum. DNA transfections were by the calcium phosphate procedure, and included a neomycin-resistance gene expression vector, pDOJ (Bloch et al., 1989), and a 5-fold excess of a GAP-43 expression vector or pCDM8 (Zuber et al., 1989a). Stable transfectants were selected in 400 μg/ml G418 and then screened for protein expression by immunoblotting. After selection, clones were maintained without G418. Those clones with the highest levels of expression were used in subsequent experiments.

Immunoblots

Particulate and soluble fractions from COS cells were analyzed by immunoblotting essentially as described previously (Zuber et al., 1989b). Forty hours after transfection, COS cells were washed with PBS and scraped from the tissue culture dish. The material was diluted in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, sonicated, and then centrifuged at 250,000 ***g*** for 30 minutes at 4°C. The supernatant was analyzed as the cytosolic fraction and the pellet was resuspended as the particulate fraction. Total protein (20 μg) from various A431 lines was separated by SDS-PAGE, transferred to nitrocellulose, and then stained for GAP-43 as described previously (Strittmatter et al., 1991).

Analysis of filopodial formation

Filopodial formation was assessed essentially as described previously (Zuber et al., 1989a). Cultures of COS-7 cells were trypsinized at 30-50% confluency with 0.25% trypsin for 5 minutes at 37°C. The cells were then diluted into at least 20 volumes of serum-containing medium and plated onto glass slides that had been precoated with poly-L-lysine at 0.1 mg/ml in PBS for 1 hour. After incubation for 8 minutes at 37°C, cells were fixed with 3.7% formaldehyde in PBS and then incubated for 1 hour in 5% normal goat serum, 0.1% Triton X-100, PBS with 1:1000 rabbit anti-GAP-43 serum, or with 1 μg/ml anti-CAT antibody (5′-3′, Inc.). Bound antibody was detected by fluorescence after incubating with fluorescein-labeled goat anti-rabbit IgG.

COS cells expressing the protein of interest were identified by fluorescence microscopy and then positive cells were scored for filopodia under Nomarski optics. Consecutive cells were scored positive or negative for any spike-like protrusion from the cell body greater than 2 μm in length. At least 50 cells were counted in each experiment, and each cell line or transient expression assay was examined in at least three separate experiments, and at least twice by an observer unaware of the identity of the transfected gene. Control cells expressing CAT were indistinguishable from non-expressing cells (data not shown).

To measure filopodia formation in the absence of substratum contact, trypsinized COS cells were maintained in suspension for 5-120 minutes at 37°C, and then centrifuged at 1000 ***g***, 4°C. The pellet was resuspended in fixative and the cells were stained as described above.

Analysis of cell spreading

For assays of spreading, cultures of COS-7 or A431 cells were trypsinized, diluted and plated, as for the filopodial assay. Glass slides were precoated with laminin at 10 μg/ml in PBS for 1 hour, or with poly-L-lysine (PLL) at 0.1 mg/ml in PBS for 1 hour. After 25 minutes

(COS cells on PLL) or 120 minutes (A431 cells on laminin) at 37°C in 5% CO_2, the medium was aspirated and the cells were fixed. Qualitatively, COS cell spreading was twice as fast as A431 cell spreading, and the spreading of both cell types on PLL was about twice as fast as on laminin. The difference between the GAP-43-transfected cells and controls was of the same magnitude regardless of the substratum. COS cells were fixed and stained as described for the filopodial assay. A431 cells were stained with 0.1% Coomassie Blue in 25% isopropanol, 10% acetic acid. The size of the stained cells was determined from micrographs of randomly chosen fields by an observer unaware of the identity of the samples. For 75 consecutive cells, the maximal and minimal diameter passing through the nucleus was recorded. The trends in the data were identical, regardless of whether maximal or minimal data were analyzed, but all data in this paper are based on the minimal diameter. Cells were classified as 'spread' if the minimal diameter exceeded 20 μm.

Determination of cAMP levels in A431 cells

A431 cells were grown to 40% confluency. Two hours prior to the assay, the cells were incubated with DMEM and no serum. To initiate cAMP accumulation, the medium was changed to 500 μM IBMX, and 20 mM Na Hepes, Hanks' balanced salt solution, pH 7.4, 0 or 50 μM forskolin, and 0 or 100 μM lysophosphatidic acid. After 10 minutes at 23°C, the cells were fixed with 2 volumes of ethanol, the material was centrifuged at 12,000 ***g*** for 10 minutes and the supernatant was evaporated to dryness. The cAMP content was determined by a radioimmunoassay method (New England Nuclear). Cyclic AMP levels were standardized for protein content analyzed from duplicate wells by the BCA method (Pierce).

RESULTS

Effect of GAP-43 on cell shape

We first confirmed that GAP-43 expression reproducibly alters COS-7 cell morphology. As described previously (Zuber et al., 1989a), filopodial extension is observed in about 20% of control COS cells, but in 55% of GAP-43-expressing COS cells under our routine conditions. To better characterize the effects of GAP-43 on non-neuronal cells, we examined cellular morphology at various times after trypsinization and replating. Before contact with a substratum allowing adhesion, about 25% of control COS-7 cells exhibit filopodia (shown as zero time point in Fig. 1A). Transfection with GAP-43 increases this to 65%. The presence of filopodia is not significantly altered by varying the length of time from 5 minutes to 120 minutes during which the cells are maintained in suspension after trypsinization (not shown). After contact with an adherent substratum the percentage of cells with filopodia rapidly declines in both the control and GAP-43-transfected cells; the rate of decline is approximately the same in both groups (Fig. 1A). Thus, the effect of GAP-43 is to alter the initial morphology after trypsinization, and not to alter the influence of the substratum on cell shape.

Filopodia are exhibited by few GAP-43-expressing COS or CHO cells after 30 minutes of incubation with an appropriate substratum. However, at this time, the shape of GAP-43 cells can still be distinguished from control cells by another attribute, the degree of cell spreading. GAP-43 expression appears to delay cell spreading. When control COS cells are plated on poly-L-lysine-coated glass, cell spreading occurs over the first 60 minutes (Fig. 1B). The time required to achieve the same degree of spreading is prolonged in the GAP-43-expressing cells, although the degree of spreading eventually reaches the same level. One day after plating, cell shape is indistinguishable between control and GAP-43 cells (not shown). Widmer and Caroni (1993) have also reported recently that cells expressing high levels of GAP-43 spread less well than controls.

The amino terminus of GAP-43 is necessary and sufficient to alter cell shape

As an initial screen for the role of different GAP-43 regions, we examined the effect of GAP-43 deletions. A large internal deletion of GAP-43 from residue 41 through 189 removes both the protein kinase C phosphorylation site (Coggins and Zwiers,

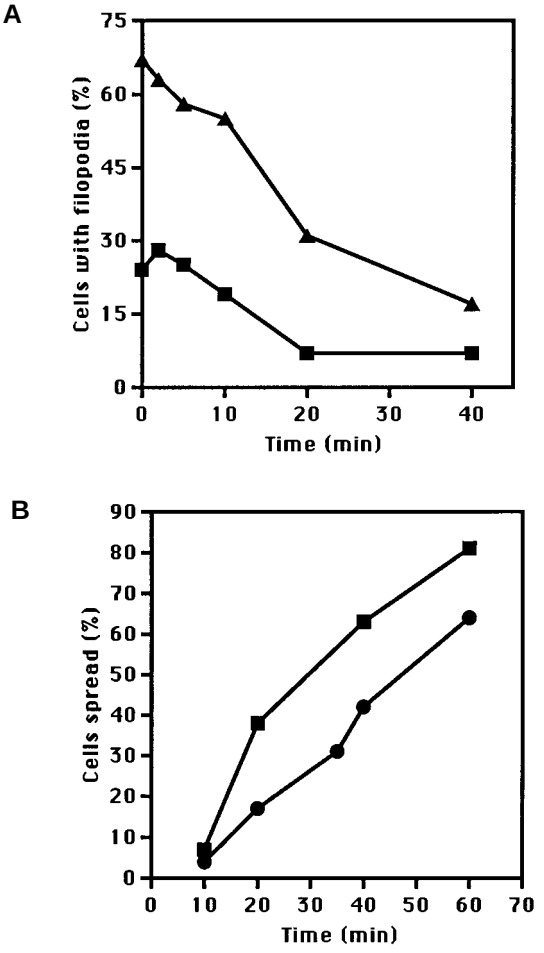

Fig. 1. Temporal changes in the morphology in GAP-43-expressing cells. (A) COS cells expressing CAT (■) or GAP-43 (▲) were analyzed for filopodial formation at various times after replating the cells on poly-L-lysine-coated glass. Note that at the shortest times examined, more GAP-43 cells have filopodia, and that few cells have filopodia after 30 minutes. The zero time point represents cells that were fixed before plating. This is one of four experiments with similar results. (B) COS cells expressing CAT (■) or GAP-43 (●) were plated for the indicated times on poly-L-lysine-coated glass, and then analyzed for the percentage of cells spread. Spreading begins after 10 minutes and is complete within 2 hours. Note that a greater percentage of spread cells is detected in the control CAT transfection. This is one of four experiments with similar results.

1989) and the calmodulin binding domain (Alexander et al., 1988). When transiently expressed in COS cells, this protein is as effective as is intact GAP-43 in enhancing filopodia and in decreasing cell spreading (Fig. 2). Thus, in these assay systems, neither phosphorylation nor calmodulin binding is absolutely necessary for GAP-43 action on cell shape. Of course, one or both activities might modulate the effectiveness of some other domain of GAP-43 in causing these changes, as suggested by the Ser42 mutations in the study of Widmer and Caroni (1993).

It is known from phylogenetic studies that the C-terminal portion of GAP-43 shows relatively little sequence conservation (LaBate and Skene, 1989), so that the activity of the internal deletion GAP-43 mutant was more likely to reside in the N-terminal, 1-40 fragment than in the C-terminal, 190-226 fragment. Therefore, we expressed a mutant GAP-43 with a deletion at the N terminus, from residue 2 through 5, a deletion that should remove both the membrane-binding (Zuber et al., 1989b; Liu et al., 1991) and the G-protein-activating function (Strittmatter et al., 1990; Sudo et al. 1992). This modified GAP-43 was devoid of activity in both cell shape assays (Fig. 2).

To test whether the N-terminal segment from 1-40 is sufficient for GAP-43 action, we expressed a chimeric protein containing an N-terminal fragment of GAP-43 fused to the N terminus of CAT in COS cells. This fusion protein also causes an increase in filopodial number and a decrease in spreading (Fig. 2). CAT alone is inactive in these assays. A series of GAP-43/CAT fusion proteins was examined to better define the active sequence. The GAP-43(1-10)/CAT protein is fully active, but the GAP-43(1-6)/CAT protein causes no change in filopodia or spreading when compared with control cells expressing CAT. The region of GAP-43 required in these assays matches that necessary to stimulate GTP binding to purified G protein (Sudo et al., 1992).

Basic residues Arg6 and Lys9 are required for cell shape modulation

Within this short active fragment of GAP-43, three of the amino acid residues are basic (Met1-Leu2-Cys3-Cys4-Met5-

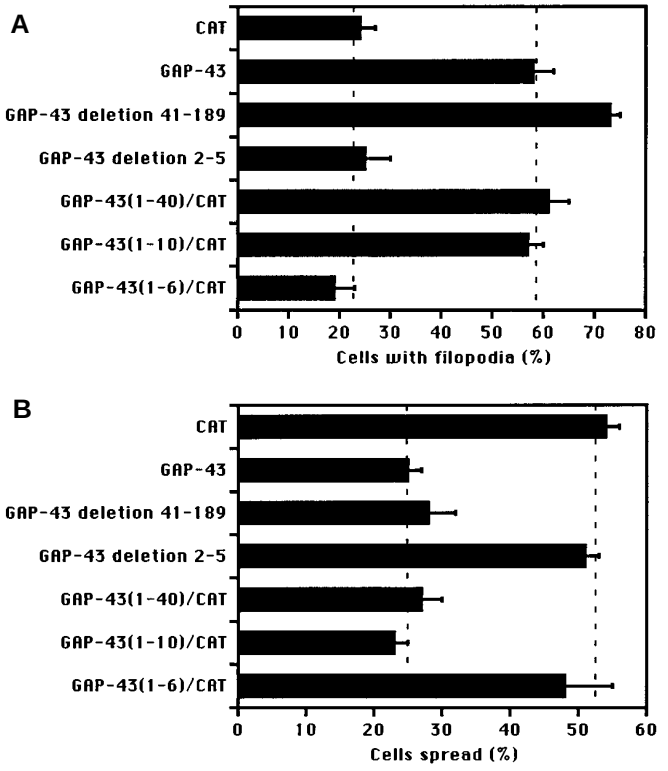

Fig. 2. The first 10 amino acids of GAP-43 are necessary and sufficient to induce cell shape changes. COS cells expressing the indicated proteins were analyzed for (A) filopodia and (B) spreading after plating. Note that GAP-43, GAP-43 with a deletion from 41-189, GAP-43(1-40)/CAT fusion and GAP-43(1-10)/CAT are active in both assays, but that GAP-43 with a deletion form 2-5 and GAP-43(1-6)/CAT are indistinguishable from the CAT control. The values shown are means ± s.e.m. for 3-7 separate experiments for each plasmid. The average cell diameter in μm for the cells analyzed in (B) was 21.2±1.2 (CAT-expressing cells), 14.3±0.8 (GAP-43), 19.6±1.0 (GAP-43 deletion 2-5) and 14.6±0.8 (GAP-43(1-40)/CAT), 14.3±0.8 (GAP-43(1-10)/CAT) and 21.3±1.0 (GAP-43(1-6)/CAT).

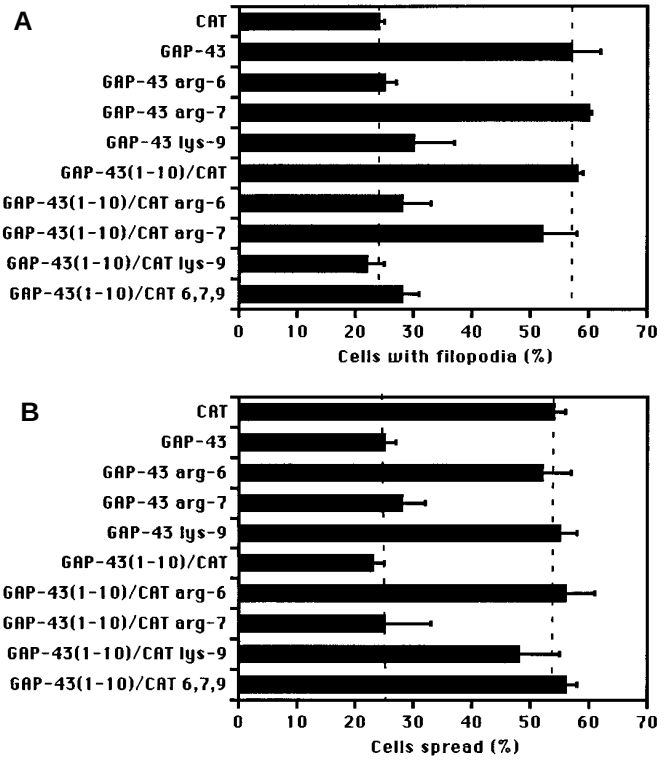

Fig. 3. Basic residues at positions 6 and 9 are required for GAP-43 modulation of cell shape. COS cells expressing the indicated CAT, GAP-43 or GAP-43(1-10)/CAT proteins were analyzed for (A) filopodia and (B) spreading. The noted amino acids are the mutated ones. Note that GAP-43, GAP-43 with Arg7 changed to Gly, GAP-43(1-10)/CAT, and the fusion protein with Arg7 changed to glycine are active in both assays. In contrast, mutants with Arg6 or Lys9 changed to Gly exhibit levels of filopodia and spreading that are the same as CAT controls. The values shown are means ± s.e.m. for 3-7 separate experiments for each plasmid. The average cell diameter in μm for the cells analyzed in (B) was 21.2±1.2 (CAT-expressing cells), 14.3±0.8 (GAP-43), 20.8±1.1 (GAP-43 arg-6), 16.1±0.9 (GAP-43 arg-7) and 22.9±1.2 (GAP-43 lys-9).

Arg6-**Arg7**-Thr8-**Lys9**-Gln10). Because a similar array of basic residues (BBxB) is present in the canonical G protein activator domain of receptors (Okamoto et al., 1990, 1991a), we tested the importance of these residues for GAP-43 action. Substitution of glycine for Arg6 or Lys9 completely blocks the action of GAP-43(1-10)/CAT and GAP-43 on cell shape (Fig. 3). In contrast, substitution for Arg7, which results in a molecule with the same amino acid composition but a different sequence from the Arg6 substitution, does not abrogate GAP-43 or fusion protein effects. All double and triple basic residue substitutions in the GAP-43(1-10)/CAT protein also are inactive (only the triple basic substitution is shown, Fig. 3). Thus, there is a specific sequence requirement in this region for two of the three basic sites. Because the GAP-43(1-10)/CAT proteins do not contain the GAP-43 protein kinase C phosphorylation site, the inactivity of the Arg6 and Lys9 mutants cannot be explained by altered phosphorylation.

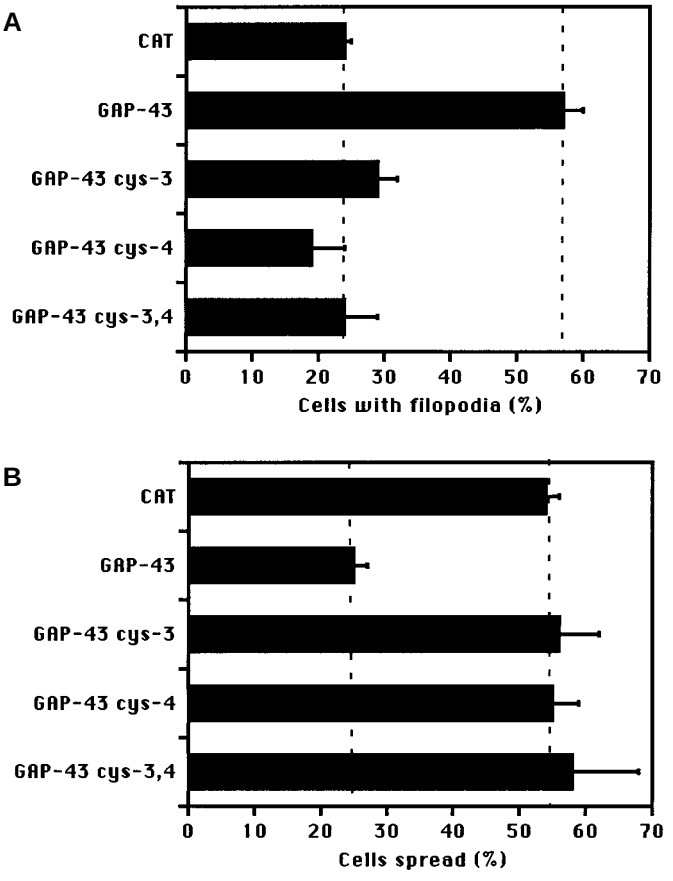

Fig. 4. Cysteine residues at positions 3 and 4 are required for the morphoregulatory activity of GAP-43. COS cells expressing the indicated proteins were analyzed for (A) filopodia and (B) spreading. Note that GAP-43 is active in both assays, but that GAP-43 with one or both cysteines at positions 3 and 4 changed to threonine is the same as the CAT control. The values shown are means ± s.e.m. for 3-7 separate experiments for each plasmid. The average cell diameter in μm for the cells analyzed in (B) was 21.2±1.2 (CAT-expressing cells), 14.3±0.8 (GAP-43), 21.9±1.0 (GAP-43 cys-3), 22.5±0.9 (GAP-43 cys-4) and 21.1±0.9 (GAP-43 cys-3,4).

Cysteine residues Cys3 and Cys4 are required for GAP-43 activity

In addition to the cluster of basic residues, the amino terminus of GAP-43 contains two adjacent cysteine residues at positions 3 and 4. These cysteines have been reported to undergo reversible cycles of palmitoylation (Skene and Virag, 1989), and they are required for the membrane binding of the protein (Zuber et al., 1989b; Liu et al., 1991). In addition, these cysteines are important for G protein activation by GAP-43. Palmitoylation of these residues reversibly

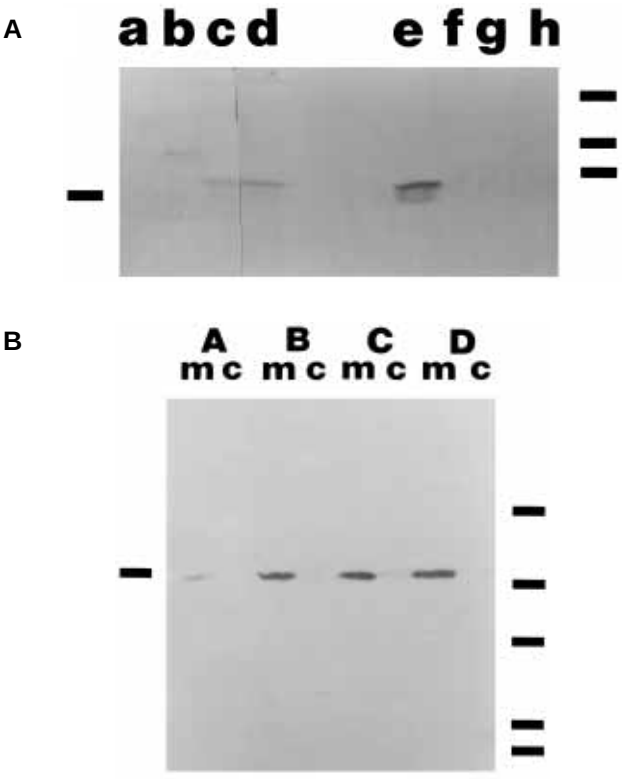

Fig. 5. Inactive mutant proteins can still bind to the membrane. (A) GAP-43/CAT fusion protein without cell shape modulating activity is localized in membrane fractions. COS cells expressing CAT (a,e), GAP-43(1-40)/CAT (b,f), GAP-43(1-10)/CAT (c,g) or GAP-43 (1-6)/CAT (d,h) were separated into membrane (a,b,c,d) and soluble (e,f,g,h) fractions and analyzed for CAT immunoreactivity on blots of 12% SDS-PAGE gels. Membrane and soluble fractions from 10^6 cells were loaded in each lane. Note that all three fusion proteins are detected in the membrane fraction but CAT is in the cytosolic fraction. The migration of Coomassie Blue-stained CAT protein is indicated by the bar at the left, and the migration of molecular mass markers of 36, 29 and 24 kDa is shown at the right. (B) Basic residues at positions 6, 7 and 9 of GAP-43 are not required for membrane binding. COS cells expressing GAP-43 (A), GAP-43 with Arg6 changed to Gly (B), GAP-43 with Arg7 changed to Gly (C), or GAP-43 with Lys9 changed to Gly (D) were separated into membrane (m) and cytosolic (c) fractions and analyzed by immunoblotting for GAP-43 immunoreactivity after 10% SDS-PAGE. Note that all four proteins are present primarily in the membrane fraction. Material from 10^6 cells was loaded in each lane. The migration of Coomassie Blue-stained GAP-43 protein is indicated by the bar at the left, and the migration of molecular mass markers of 66, 45, 36, 29 and 24 kDa is shown at the right.

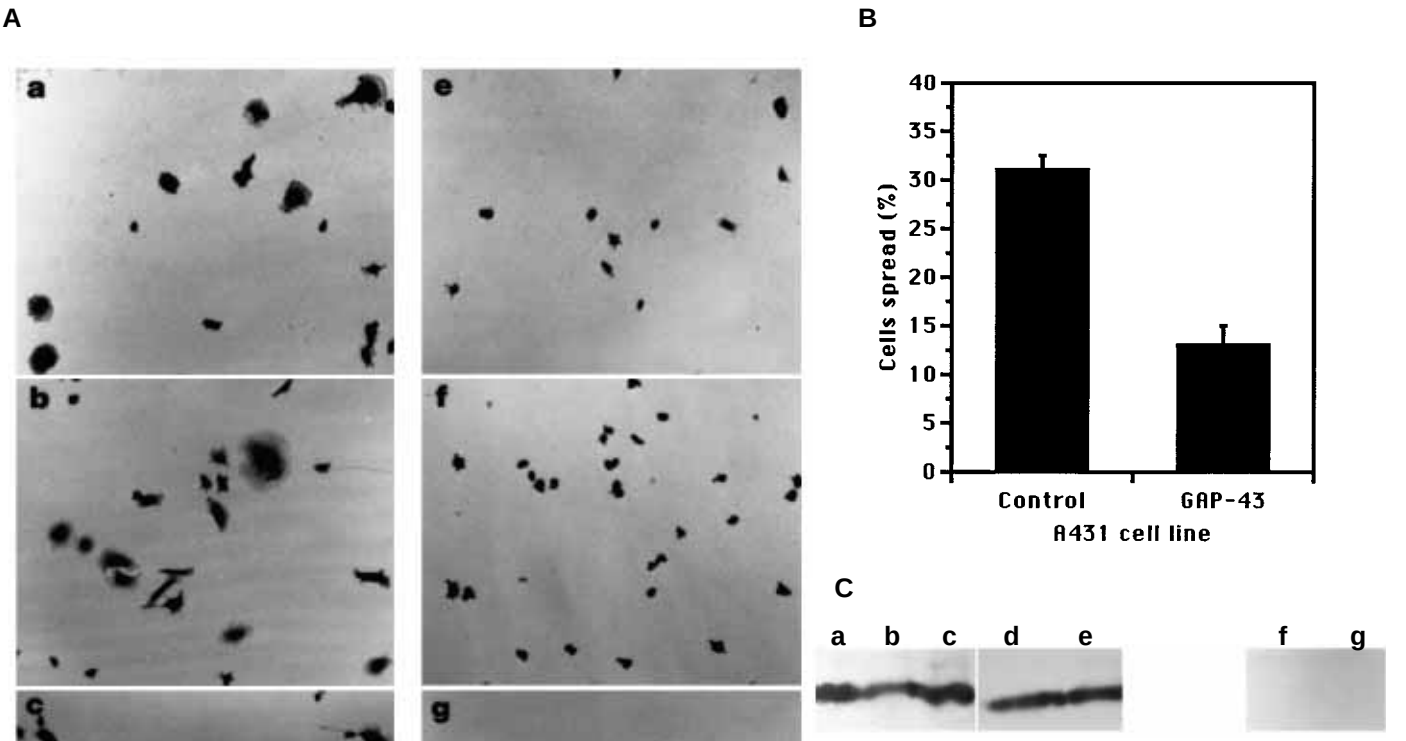

Fig. 6. Decreased spreading of A431 cells stably transfected with GAP-43. (A) Four control A431 cell lines (a,b,c,d) exhibit a more flattened, spread phenotype as compared to four GAP-43-expressing A431 cell lines (e,f,g,h). These cells were fixed 2 hours after plating on laminin-coated glass. Bar, 50 μm. (B) Control or GAP-43-expressing A431 cell lines were assayed for cell spreading on laminin-coated glass. The data are averaged from 6 control and 7 GAP-43 cell lines, and the s.e.m. is shown. The average cell diameter was 23.6±0.8 μm for the control cells and 15.8±0.7 μm for the GAP-43-expressing cells. (C) Separate A431 cell lines transfected with a GAP-43 expression vector (a,b,c,d,e), or with control plasmid (f,g) were analyzed for GAP-43 expression by immunoblotting. Note that control cells do not express GAP-43 but that the transfected cells do so. The level of GAP-43 expression in these cell lines per mg total protein is 10-50% of that in neonatal brain (not shown).

blocks interaction of GAP-43 and G_o (Sudo et al., 1992), and a GAP-43(1-10) peptide with threonines substituted for cysteines does not activate G_o (Strittmatter et al., 1990). If one or both of these activities are important for the GAP-43-induced alteration of cell morphology, then mutation of the cysteines should prevent GAP-43 action. Indeed, deletion of the cysteines and two adjacent residues does inactivate GAP-43 (Fig. 2). To delineate the importance of each of the cysteines, GAP-43 molecules with point mutations of the cysteines were expressed in COS cells. These proteins have no effect on filopodia or spreading (Fig. 4). This is consistent with the observations of Widmer and Caroni (1993) that a Cys3Cys4 to Ala3Ala4 GAP-43 mutant does not alter COS cell shape.

Inactive mutants are associated with particulate fractions

Since the N terminus of GAP-43 also directs membrane binding (Zuber et al., 1989b; Liu et al., 1991), mutants of this region may fail to alter cell shape due to altered subcellular distributions. Therefore, we determined whether the inactive N-terminal mutants were present in soluble or particulate fractions. As demonstrated previously (Zuber et al., 1989b), the GAP-43(1-40)/CAT and the GAP-43(1-10)/CAT fusion proteins are localized predominantly to particulate fractions, as opposed to the soluble CAT molecule (Fig. 5). The inactive GAP-43(1-6)/CAT fusion protein is detectable primarily in particulate fractions, as are GAP-43 mutants with the single basic amino acid substitutions (Fig. 5). The particulate local-

ization of three inactive proteins, GAP-43(1-6)/CAT, Arg6-substituted GAP-43, and Lys9-substituted GAP-43, demonstrates that their inactivity is not due to interference with subcellular localization.

GAP-43 decreases forskolin-stimulated cAMP levels

GAP-43 can activate G_o and G_i, but not G_s (Strittmatter et al., 1991), and GAP-43 amino-terminal peptides also activate G_o (Sudo et al., 1992). Thus, it is reasonable to predict that GAP-43 might alter G protein transduction after transfection into non-neuronal cells. To assess this hypothesis, it was necessary to obtain a population of cells uniformly expressing GAP-43, for which we utilized stable transfectants of A431 cells. This cell line has a well-characterized adenylate cyclase system (Guillet et al., 1985) and, unlike COS-7 cells, can be stably transfected with expression vectors derived from pCDM-8. GAP-43-expressing A431 cells exhibit decreased cell spreading (Fig. 6), as shown for COS-7 cells and L6 cells (Fig. 1B; Widmer and Caroni, 1993). Since A431 cells contain G_i (but not G_o), GAP-43 might be predicted to inhibit adenylate cyclase. We assessed the adenylate cyclase system in cells transfected with GAP-43 as compared to control A431 cell lines. The basal level of cAMP accumulation upon the addition of IBMX is low and not altered by the presence of GAP-43. Forskolin, which directly augments cyclase activity, elevates cAMP levels 100-fold in control cell lines (Fig. 7), but only about 50-fold in GAP-43-expressing cell lines. The two simplest explanations for this finding are that G_i is activated by GAP-43, or, alternatively, that adenylate cyclase itself is underexpressed in the GAP-43 cells. To distinguish between these possibilities, we measured cAMP levels in the presence of forskolin and lysophosphatidic acid (LPA), which has been shown to inhibit adenylate cyclase by binding to a receptor that activates G_i (Murayama and Ui, 1987; van Corven et al., 1989).

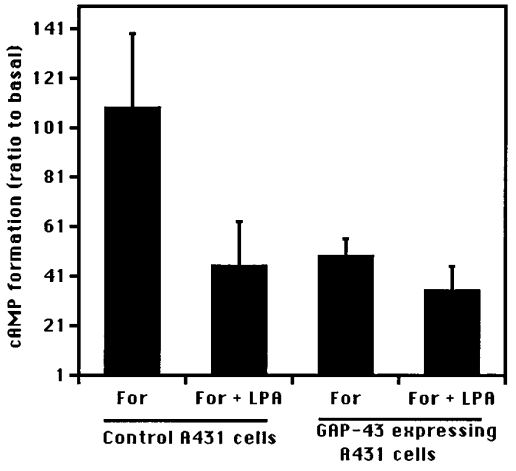

Fig. 7. GAP-43 decreases forskolin-stimulated cAMP within A431 cell lines. The accumulation of cAMP in control A431 cell lines and GAP-43-expressing cell lines was determined in the presence of forskolin (For) or forskolin plus lysophosphatidic acid (LPA) as described in Materials and Methods. The s.e.m. are from four to six separate cell lines. The values are expressed as a ratio to the basal value, which was 37±6 pmol/mg protein per 15 minutes for the control cells and 43±12 in the GAP-43-expressing cells.

The addition of LPA reduces the cAMP level in control cells to that of the GAP-43 cells, whereas LPA causes no significant reduction in cAMP levels in the GAP-43-expressing cells. This argues that G_i is fully activated in the GAP-43-expressing cells, so that the addition of LPA is incapable of further altering the cAMP level. Identical changes in cAMP accumulation have been observed in cells transfected with a constitutively activated oncogenic mutant of G_i (Wong et al., 1991).

Isoproterenol and lysophosphatidic acid modulate cell shape

If these changes in cAMP levels are responsible for GAP-43 effects on non-neuronal cell shape, increases in cAMP should antagonize GAP-43 effects, and inhibition of adenylate cyclase should cause changes similar to those of GAP-43. To test these predictions, isoproterenol and LPA were added to control and GAP-43-expressing COS cells. Isoproterenol is a β_2-adrenergic receptor agonist that activates G_s and stimulates adenylate cyclase. In fibroblasts, LPA inhibits adenylate cyclase through a receptor linked to G_i, and also activates G-protein-dependent phosphoinositide hydrylosis and arachadonic acid release (van Corven et al., 1989). In non-transfected COS cells, LPA increases the percentage of cells with filopodia to a level similar to that induced by GAP-43 transfection (Fig. 8A). LPA also inhibits spreading to the same degree as GAP-43 (Fig.

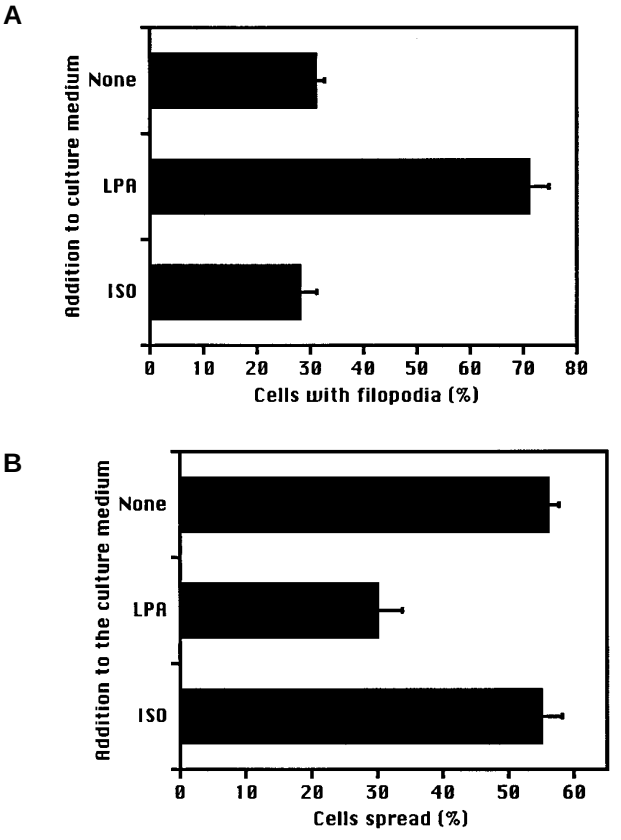

Fig. 8. LPA increases filopodia and decreases spreading in COS cells. COS cells were plated in the presence of no addition, 100 μM LPA or 1 μM isoproterenol (ISO) and analyzed for filopodia (A) and spreading (B) as described in Materials and Methods. The drugs were added immediately before plating. The values illustrated are the means ± s.e.m.for 4-12 independent measurements.

8B). Isoproterenol has little effect on filopodia or spreading in non-transfected cells (Fig. 8). COS cells expressing GAP-43 respond in a different fashion (Fig. 9). LPA has no significant effect on cell shape, but isoproterenol decreases the percentage of filopodia-positive cells and increases cell spreading to the levels observed in the basal state. The ability of isoproterenol to reverse the effect of GAP-43 on cell spreading is also observed in stably transfected A431 cells (Fig. 10). The effects of GAP-43, LPA and isopreterenol on cell shape were observed without the IBMX and forskolin present in the cAMP assays. These agents were added to the cAMP assays to produce easily detectable signals, but were not present in the cell shape assays, in order to avoid the possibility of saturating effects during an 8-60 minutes incubation at 37°C (as opposed to 10 minutes at 23°C for cAMP assays).

DISCUSSION

We report here that the first ten amino acids of GAP-43 are critical for its alteration of cell shape. The activities of GAP-43 and of GAP-43/CAT chimeric proteins are sensitive to point mutations of Cys3, Cys4, Arg6 and Lys9, but not of Arg7. Other segments of the GAP-43 molecule do not appear to be absolutely essential for GAP-43 action on cell shape. The Ser41 phosphorylation site clearly has an important regulatory role in this cell shape assay, since point mutations mimicking phosphorylated GAP-43 (Asp42 of chick GAP-43) spread more than do those expressing wild-type GAP-43 (Widmer and Caroni, 1993). It may be that the phosphorylation site modulates the efficacy of the amino-terminal domain, or, alternatively, a domain including the phosphorylation site may have an independent action on cell morphology. Such a second domain with an independent morphoregulatory function has not yet been defined, but the calmodulin-binding domain of GAP-43 (Alexander et al., 1988) is one possibility.

No direct extrapolation should be made from the effects of GAP-43 upon the shape of non-neuronal cells to the morphology of growth cones, but we (Zuber et al., 1989) and others (Widmer and Caroni, 1993) have used this system because there is no direct assay for GAP-43 action in neuronal cells that might allow analyses of protein domains. It is not inconceivable that the assay has relevance to neurons, since regulation of membrane and cytoskeletal dynamics is to some extent similar in all eukaryotic cells (Smith, 1988; Bray and Hollenbeck, 1988; Bray and White, 1988).

The amino-terminal domain critical for the cellular activities reported here has been evaluated in detail previously with regard to GAP-43 stimulation of G_o and G_i. GAP-43 and GAP-43(1-10) peptides stimulate G_o, unless the cysteines are oxidized or palmitoylated (Sudo et al., 1992). Although there are other potential explanations for the GAP-43 effect on non-neuronal cell shape, the sequence similarities led us to speculate that the cell shape effects involve changes in G protein activity. For that reason, we examined the adenylate cyclase system of stably transfected A431 cells. GAP-43-expressing cells exhibit decreased forskolin-stimulated cAMP levels, a finding that is most simply explained by persistent G_i activation. These measurements do not demonstrate direct stimulation of G_i by GAP-43. The ability of physiological concentrations of GAP-43 to stimulate other G protein transduction systems has been demonstrated recently by the microinjection of GAP-43 into *Xenopus laevis* oocytes (Strittmatter et

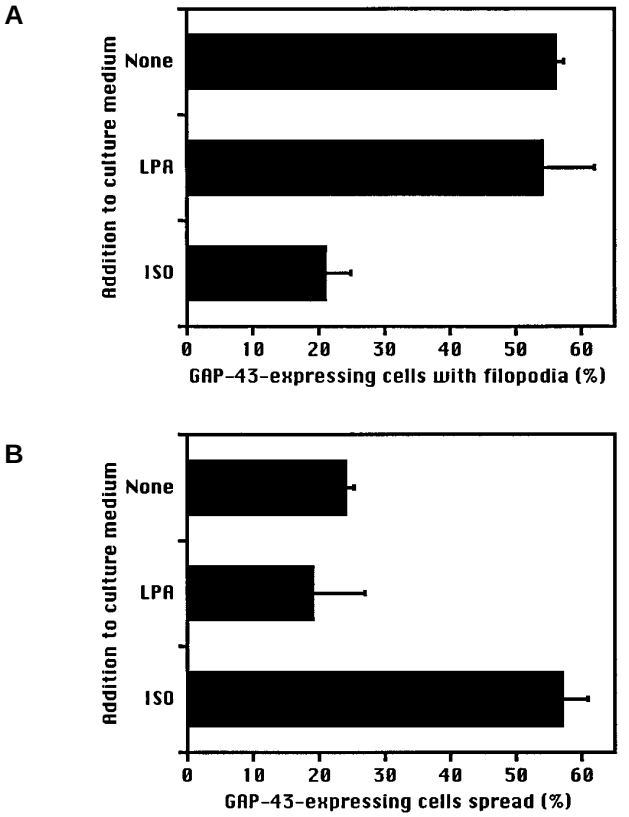

Fig. 9. Isoproterenol reverses the effect of GAP-43 on COS cell morphology. COS cells were transfected with GAP-43 expression vector and after 40 hours the cells were replated in the absence of drug, or the presence of 100 μM LPA or 1 μM isoproterenol (ISO). Those cells expressing GAP-43 were analyzed for filopodia (A) and spreading (B). The means ± s.e.m.for 3-12 experiments are shown.

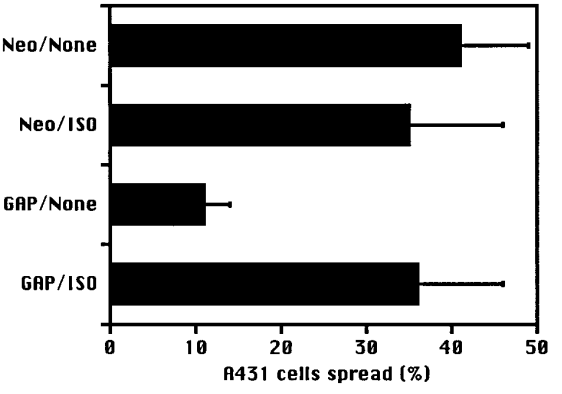

Fig. 10. Isoproterenol prevents GAP-43 inhibition of A431 cell spreading. The percentage of stably transfected control (Neo) or GAP-43-expressing (GAP) A431 cells that were spread was determined after a 2 hour incubation on laminin-coated glass in the presence of 100 μM LPA or 1 μM isoproterenol (ISO). The means ± s.e.m. are from four to six separate cell lines.

al., 1993). In that case, GAP-43 stimulates a G protein cascade involving inositol trisphosphate formation and the release of intracellular calcium. Thus, there is evidence that GAP-43 can alter the function of both adenylate cyclase and phospholipase-C-dependent pathways.

G protein perturbation has been documented to modulate cell shape in many cells, including fibroblasts (Klebe et al., 1991), macrophages (Petty and Martin, 1989), and neutrophils (Bengtsson et al., 1990). LPA activates several G protein signaling pathways in fibroblasts (van Corven et al., 1989), and mimics GAP-43 action on cell shape in COS cells. The ability of isoproterenol to block GAP-43-induced cell shape changes demonstrates that inhibition of adenylate cyclase through G_i activation could account for GAP-43-induced alterations in filopodia and spreading. Of course, other signalling pathways could also contribute to the effect of GAP-43.

There is direct evidence that G-protein transduction systems have a major role in growth cone control. Serotonin (Haydon et al., 1984), dopamine (Lankford et al., 1988; Rodrigues and Dowling, 1990) and thrombin (Suidan et al., 1992), all of which act via G-protein-coupled receptors, cause growth collapse and the cessation of neurite elongation. Growth cone collapse induced by membrane-bound, target-derived proteins is mediated by a pertussis toxin-sensitive G-protein (Igarashi et al., 1992). The outgrowth-promoting effects of NCAM and N-cadherin are blocked by pertussis toxin (Doherty et al., 1991). Since both growth-promoting and growth-inhibiting factors can act via G protein cascades, the particular mix of receptors, G proteins and second messenger systems in different neurons will determine whether their net growth response to particular ligands is positive or negative.

Specific amino acids are critical to the GAP-43 effect on cell shape. Previously we had noted sequence similarities between the G-protein-activating amino terminus of GAP-43 and the cytoplasmic tail of these receptors, consisting of a hydrophobic-Leu-Cys-Cys-x-x-basic-basic motif (Strittmatter et al., 1990). Although there is still debate about precisely which regions of the seven-spanning receptors interface with G proteins (reviewed by Ross, 1989), more recent data have drawn attention to a stretch of the third cytosolic loop, which contains a basic-basic-x-x-basic, or basic-basic-x-basic motif with adjacent basic residues (Okamoto et al., 1990, 1991a). For G protein activation, the first and third basic residues are critical, but the second is not (Okamoto et al., 1991b). The G-protein-activating region of GAP-43 does include the stretch Arg6-Arg7-x-Lys9. The morphoregulatory activity of this region is diminished by mutations of Arg6 and Lys9, but not of Arg7. Thus, over this short stretch, the basic amino acid sequence constraints of GAP-43 bear a similarity to those of a G-protein-activating domain of receptors. Although it lacks the adjacent basic residues, it is conceivable that the α-amino group of GAP-43 could provide an additional positive change if it is not blocked in vivo (Wakim et al., 1987). Hence, the amino terminus of GAP-43 may exhibit G protein activator function based on its homology to either this basic third cytoplasmic loop of receptors and/or the cytoplasmic tail of receptors. Alternatively, because this region is so short, these similarities may be fortuitous, and GAP-43 could stimulate G proteins by an unrelated mechanism.

The present study identifies a short stretch of GAP-43 as having morphoregulatory activity. This is of interest in light of speculations concerning the role of GAP-43 in growth cones and in synaptic plasticity (Benowitz and Routtenberg, 1987; Skene, 1989; Strittmatter et al., 1992), both of which involve focal cell shape changes (Bray and Hollenbeck, 1998; Bailey and Chen, 1988). The GAP-43 sequence identified here can stimulate isolated G_o (Sudo et al., 1992), so these experiments might be interpreted as suggesting that GAP-43 modulates cell shape by intracellular regulation of G protein transduction. In order to evaluate this thesis directly in neurons, it will be necessary to generate normal nerve cells devoid of native GAP-43, a process that can be accomplished most convincingly by gene ablation studies employing homologous recombination.

We thank Xaioping Zhu, Margaret Boulos and Ling Li for extensive and expert technical assistance, Kerry Ressler for performing immunoblots to screen some A431 clones, and Mauricio Zuber for some of the GAP-43 and GAP-43 mutant expression vectors. This work is supported by a NIH-CIDA (to S.M.S.) and by a grant from Sumitomo Pharmaceutical and Chemical Cos. (to M.C.F.).

REFERENCES

Alexander, K. A., Cimler, B. M., Meier, K. E. and Storm, D. R. (1987). Regulation of calmodulin binding to P-57. *J. Biol. Chem.* **262**, 6108-6113.

Alexander, K. A., Wakim, B. T., Doyle, G. S., Walsh, K. A. and Storm, D. R. (1988). Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. *J. Biol. Chem.* **263**, 7544-7549.

Baetge, E. E. and Hammang, J. P. (1991). Neurite outgrowth in PC12 cells deficient in GAP-43. *Neuron* **6**, 21-30.

Bailey, C. H. and Chen, M. (1988). Long-term sensitization in *Aplysia* increases the number of presynaptic contacts onto the identified gill motor neuron L7. *Proc. Nat. Acad. Sci. USA* **85**, 9356-9359.

Bauer, P. A., Muller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E. J. M. and Lohse, M. J. (1992). Phosducin is a protein kinase A-regulated G-protein regulaotr. *Nature* **342**, 73-76.

Bengtsson, T., Sarndahl, E., Stendahl, O. and Andersson, T. (1990). Involvement of GTP-binding proteins in actin polymerization in human neutrophils. *Proc. Nat. Acad. Sci. USA* **87**, 2921-2925.

Benowitz, L. I. and Routtenberg, A. (1987). A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism and synaptic plasticity. *Trends Neurosci.* **10**, 527-532.

Bloch, D. B., Bonventre, J. V., Neer, E. J. and Seidman, J. G. (1989). The G protein α_o subunit alters morphology, growth kinetics and phospholipid metabolism of somatic cells. *Mol. Cell. Biol.* **9**, 5434-5439.

Bray, D. and Hollenbeck, P. J. (1988). Growth cone motility and guidance. *Annu. Rev. Cell Biol.* **4**, 43-61.

Bray, D. and White, J. G. (1988). Cortical flow in animal cells. *Science* **239**, 883-888.

Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A. and Storm, D. R. (1991). Characterization of the calmodulin binding domain of neuromodulin. *J. Biol. Chem.* **266**, 207-213.

Coggins, P. J. and Zwiers, H. (1989). Evidence for a single protein kinase C phosphorylation site in rat brain protein B-50. *J. Neurochem.* **53**, 1895-1901.

Doherty, P., Ashton, S. V., Moore, S. E. and Walsh, F. S. (1991). Morphoregulatory activities of NCAM and N-Cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca^{2+} channels. *Cell* **67**, 21-33.

Edmonds, B. T., Moonmaw, C. R., Hsu, J. T., Slaughter, C. and Ellis, L. (1990). The p38 and p34 polypeptides of growth cone particle membranes are the a- and b-subunits of G proteins. *Dev. Brain Res.* **56**, 131-136.

Fishman, M. C. (1989). Genes of neuronal plasticity. In *The Assembly of the Nervous System* (ed. L. T. Landmesser), pp. 247-257. New York: Alan R. Liss, Inc.

Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**, 615-649.

Guillet, J. G., Kaveri, S. V., Durieu, O., Delavier, C., Hoebeke, J. and

Strosberg, A. D. (1985). β-Adrenergic agonist activity of a monoclonal anti-idiotypic antibody. *Proc. Nat. Acad. Sci. USA* **82**, 1781-1784.

Haydon, P. G., McCobb, D. P. and Kater, S. B. (1984). Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561-564.

Igarashi, M., Strittmatter, S. M., Vartanian, T. and Fishman, M. C. (1992). Mediation by G proteins of signals that cause colllapse of growth cones. *Science* **259**, 77-79.

Klebe, R. J., Overfelt, T., Magnuson, V. L., Stefensen, B., Chen, D. and Zardeneta, G. (1991). Quantitative assay for morphogenesis indicates the role of extracellular matrix components and G proteins. *Proc. Nat. Acad. Sci. USA* **88**, 9588-9592.

LaBate, M. E. and Skene, J. H. (1989). Selective conservation of GAP-43 structure in vertebrate evolution. *Neuron* **3**, 299-310.

Lankford, K. L., DeMello, F. G. and Klein, W. L. (1988). D_1-type dopamine receptors inhibit growth cone motility in cultured retina neurons: Evidence that neurotransmitters act as morphogenic growth regulators in the developing central nervous system. *Proc. Nat. Acad. Sci. USA* **95**, 2839-2843.

Liu, Y., Chapman, E. R. and Storm, D. R. (1991). Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultures of rat embryonic neurons. *Neuron* **6**, 411-420.

Lockerbie, R. O. (1987). The neuronal growth cone: A review of its locomotory, navigational and target recognition capabilities. *Neuroscience* **20**, 719-729.

Meiri, K. F. and Gordon-Weeks, P. R. (1990). GAP-43 in growth cones associated with areas of membrane that are tightly bound to the substrate and is a component of the membrane skeleton subcellular fraction. *J. Neurosci.* **10**, 256-266.

Murayama, T. and Ui, M. (1987). Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J. Biol. Chem.* **262**, 5522-5529.

Oestreicher, A. B., Van Dongen, C. J., Zwiers, H. and Gispen, W. H. (1983). Affinity-purified anti-B-50 protein antibody: interference with the function of the phosphoprotein B-50 in synaptic plasma membranes. *J. Neurochem.* **41**, 331-340.

Okamoto, T., Katada, T., Murayama, Y., Ui, M. and Nishimoto, I. (1990). A simple structure encodes G protein-activating function of the IGF II/mannose 6-phosphate receptor. *Cell* **62**, 709-717.

Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E. and Nishimoto, I. (1991a). Identification of a G_s activator region of the b_2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. *Cell* **67**, 723-730.

Okamoto, T., Ohkumi, Y., Ogata, E. and Nishimoto, I. (1991b). Distinct mode of G protein activation due to single residue substitution of active IGF-II receptor peptide Arg2410-Lys2423: Evidence for stimulation acceptor region other than C-terminus of Gia. *Biochem. Biophys. Res. Commun.* **179**, 10-16.

Petty, H. R. and Martin, S. M. (1989). Combinative ligand-receptor interactions: effects of cAMP, epinephrine and met-enkephalin on RAW264 macrophage morphology, spreading, adherence, and microfilaments. *J. Cell Physiol.* **138**, 247-256.

Rodrigues, P. S. and Dowling, J. E. (1990). Dopamine induces neurite retraction in retinal horizontal cells via diacylglyceraol and protein kinase C. *Proc. Nat. Acad. Sci. USA* **87**, 9693-9697.

Ross, E. M. (1989). Signal sorting and amplification through G protein-coupled receptors. *Neuron* **3**, 141-152.

Shea, T. B., Perrone-Bizzozero, N. I., Beermann, M. L. and Benowitz, L. I. (1991). Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuritogenesis. *J. Neurosci.* **11**, 1685-1690.

Skene, J. H. P. (1989). Axonal growth-associated proteins. *Annu. Rev. Neurosci.* **12**, 127-156.

Skene, J. H. P. and Virag, I. (1989). Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *J. Cell Biol.* **108**, 613-624.

Smith, S. J. (1988). Neuronal cytomechanics: The actin-based motility of growth cones. *Science* **242**, 708-715.

Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J. and Fishman, M. C. (1990). G_o is a major growth cone protein subject to regulation by GAP-43. *Nature* **344**, 836-841.

Strittmatter, S. M. and Fishman, M. C. (1991). The neuronal growth cone as a specialized transduction system. *BioEssays* **13**, 127-134.

Strittmatter, S. M., Valenzuela, D., Sudo, Y., Linder, M. E. and Fishman, M. C. (1991). An intracellular guanine nucleotide release protein for G_o. *J. Biol. Chem.* **266**, 22465-22471.

Strittmatter, S. M., Vartanian, T. and Fishman, M. C. (1992). GAP-43 as a neuronal plasticity protein in neuronal form and repair. *J. Neurobiol.* **23**, 507-520.

Strittmatter, S. M., Cannon, S. C., Ross, E. M., Higashijima, T. and Fishman, M. C. (1993). GAP-43 augments G protein-coupled receptor transduction in *X. laevis* oocytes. *Proc. Nat. Acad. Sci. USA* **90**, 5327-5331.

Sudo, Y., Valenzuela, D., Beck-Sickinger, A. B., Fishman, M. C. and Strittmatter, S. M. (1992). Palmitoylation alters protein activity: Blockade of G_o stimulation by GAP-43. *EMBO J.* **11**, 2095-2102.

Suidan, H. S., Stone, S. R., Hemmings, B. A. and Monrad, D. (1992). Thrombin causes neurite retraction in neuronal cells through activation of cell surface receptors. *Neuron* **8**, 363-375.

van Corven, E. J., Groenik, A., Jalink, K., Eicholtz, T. and Moolenaar, W. H. (1989). Lyosphosphatidate-induced cell proliferation: identification and dissection of signalling pathways mediated by G proteins. *Cell* **59**, 45-54.

Widmer, F. and Caroni, P. (1993). Phosphorylation-site mutations of the amino terminus of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* **120**, 503-512.

Wakim, B. T., Alexander, K. A., Masure, H. B., Cimler, B. M., Storm, D. R. and Walsh, K. A. (1987). Amino acid sequence of p-57, a neurospecific calmodulin binding protein. *Biochemistry* **26**, 7466-7470.

Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouysssegur, J. and Bourne, H. R. (1991). Mutant α subunits of G_{i2} inhibit cyclic AMP accumulation. *Nature* **351**, 63-65.

Yankner, B. A., Benowitz, L. I., Villa-Komaroff, L. and Neve, R. L. (1990). Transfection of PC12 cells with the human GAP43 gene: effects on neurite outgrowth and regeneration. *Mol. Brain Res.* **7**, 39-44.

Zuber, M. X., Goodman, D. W., Karns, L. R. and Fishman, M. C. (1989a). The neuronal growth-associated protein GAP-43 induces filopodia in non-neuronal cells. *Science* **244**, 1193-1195.

Zuber, M. X., Strittmatter, S. M. and Fishman, M. C. (1989b). A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43. *Nature* **341**, 345-348.

(Received 19 July 1993 - Accepted 27 September 1993)