GAP43, MARCKS, and CAP23 Modulate PI(4,5)P₂ at Plasmalemmal Rafts, and Regulate Cell Cortex Actin Dynamics through a Common Mechanism

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Abstract. The dynamic properties of the cell cortex and its actin cytoskeleton determine important aspects of cell behavior and are a major target of cell regulation. GAP43, myristoylated alanine-rich C kinase substrate (MARCKS), and CAP23 (GMC) are locally abundant, plasmalemma-associated PKC substrates that affect actin cytoskeleton. Their expression correlates with morphogenic processes and cell motility, but their role in cortex regulation has been difficult to define mechanistically. We now show that the three proteins accumulate at rafts, where they codistribute with PI(4,5)P₂, and promote its retention and clustering. Binding and modulation of PI(4,5)P₂ depended on the basic effector domain (ED) of these proteins, and constructs lacking the ED functioned as dominant inhibitors of plasmalemmal PI(4,5)P₂ modulation. In the neuronlike cell line, PC12, NGF- and substrate-induced peripheral actin structures, and neurite outgrowth were greatly augmented by any of the three proteins, and suppressed by ΔED mutants. Agents that globally mask PI(4,5)P₂ mimicked the effects of GMC on peripheral actin recruitment and cell spreading, but interfered with polarization and process formation. Dominant negative GAP43(ΔED) also interfered with peripheral nerve regeneration, stimulus-induced nerve sprouting and control of anatomical plasticity at the neuromuscular junction of transgenic mice. These results suggest that GMC are functionally and mechanistically related PI(4,5)P₂ modulating proteins, upstream of actin and cell cortex dynamics regulation.

Key words: neurite outgrowth • cell spreading • anatomical plasticity • actin recruitment • lipid micro-domain

Introduction

Signaling at the cell surface integrates specific contextual information from the local environment by recruiting and assembling cell-specific subplasmalemmal protein complexes that regulate cell behavior. To control signal quality and strength, cells assemble signaling platforms and structures of varying composition, complexity, and stability. Actin-based structures are major components of cell-surface signaling complexes such as focal contacts, adherens junctions, caps, and supramolecular activation clusters involved in lymphocyte activation, and synapses in the nervous system (Penninger and Crabtree, 1999). Since actin assembly is an early event in the formation of these signaling structures, defining mechanisms that regulate plasmalemmal actin dynamics, and the formation of transient and stable actin-based structures can provide significant insights into cell signaling.

A t the cell surface, actin filament assembly and dynamics are subject to complex temporal and spatial control by signals from the extra- and intracellular environment (Welch et al., 1997; Schafer et al., 1998). Members of the Cdc42, Rac, and Rho family of small GTP-binding proteins couple cell-surface receptor activation to specific patterns of actin cytoskeleton regulation (Tapon and Hall, 1997). In addition, in cell-free systems, key actin binding and focal contact proteins such as gelsolin, profilin, cofillin, and vinculin are regulated by the lipid second messenger PI(4,5)P₂, which may be a critical component in promoting filament assembly at the cell membrane (Hartwig et al., 1995; Welch et al., 1997; Ma et al., 1998). However, since the overall contents of PI(4,5)P₂ at the inner leaflet of the cell membrane can be comparatively high and constant, it is not clear how this lipid second messenger regulates actin dynamics. Because of the relatively low affinities involved in these interactions, one critical parameter may be the local concentration of membrane PI(4,5)P₂ available for...
ligand binding. Accordingly, one possibility for regulation would involve the existence of mechanisms to locally concentrate and mask PI(4,5)P₂ at the inner leaflet of the cell membrane, where its accessibility to actin regulatory proteins would be linked to extracellular signals.

GAP43, myristoylated alanine-rich C kinase substrate (M A R C K S),¹ and CAP23 (G M C) are major protein kinase C (PKC) substrates associated with the plasma membrane that can bind acidic phospholipids including PI(4,5)P₂ (Skene, 1989; A derem, 1995; Seki et al., 1996; Benowitz and Routtenberg, 1997; Denison et al., 1998). Athough they do not share regions of sequence homology, GAP43 can, to a large extent, substitute for CAP23 in vivo, indicating that the two proteins are closely related functionally (Frey et al., 2000a). Mechanistically, the function of GMC is still not understood, but loss- and gain-of-function studies in cell lines and mice argue for a critical role in regulating actin-based structures and motility (Aigner and Caroni, 1995; Aigner et al., 1995; Strittmatter et al., 1995; Stumpo et al., 1995; M yat et al., 1997; Frey et al., 2000a). Such a role is also consistent with the expression of these proteins, which is highly regulated during development and in the adult, and correlates with morphogenetic processes and cell-surface dynamics (Skene, 1989; A derem, 1995; Benowitz and Routtenberg, 1997; M cNamara and L enox, 1997). GMC share several characteristic properties such as the following: membrane association mediated by acylation; colocalization at unique subplasmalemmal patches; similar highly hydrophilic amino acid compositions; and the presence of a basic effector domain that binds acidic phospholipids, calmodulin, actin filaments, and PKC (Fig. 1 A; Hartwig et al., 1992; Aderem, 1995; Benowitz and Routtenberg, 1997; He et al., 1997; Wiederkehr et al., 1997). In artificial phospholipid vesicles, the effector domains (EDs) of MARCKS and GAP43 can bind and regulate the accessibility of acidic phospholipids, including PI(4,5)P₂, through electrostatic interactions (see e.g., Deniso et al., 1998). However, presently, experimental evidence that MARCKS and GAP43 regulate lipid second messengers at the cell membrane is lacking.

In this study, we present experimental evidence that GMC sequester and modulate PI(4,5)P₂ at cholesterol-dependent microdomains. Constructs lacking the ED acted as dominant inhibitors of microdomain PI(4,5)P₂ accumulation. The effects of GMC constructs on raft PI(4,5)P₂ peripheral actin recruitment, and neurite outgrowth were highly correlated, and were comparable to those of agents that directly affect PI(4,5)P₂. Based on these results, we propose that GMC are mechanistically related membrane-associated proteins that mediate calcium- and PKC-sensitive modulation of PI(4,5)P₂ at plasmalemmal microdomains, upstream of cell cortex and actin dynamics regulation, and neurite outgrowth. In the accompanying paper (Frey et al., 2000), we provide compelling evidence that GAP43 and the related protein CAP23 are related functionally in vivo, where they have common as well as unique functions in neurite outgrowth. Together, these studies establish GMC as members of a family of mechanism and functionally related proteins. Because of their shared effects on PI(4,5)P₂ modulation, we propose the collective designation of pipmodulins.

Materials and Methods

Reagents, Cell Culture, and Transgenic Mice

Inhibitor compounds and growth factors, with their final concentrations, were used as follows. N-ethylmaleimide (10 mM), N-phenylmaleimide (100 nMm), cycloheximide (5 μM), and cytochalasin D (10 μM) from Sigma Chemical Co.; LiCl (10 mM) and calcimycin (50 μM) from Fluuka A G; and U-73122 (1 μM) from Calbiochem. For transfection, cDNAs were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen). All mutant constructs were generated by conventional PCR techniques and verified by DNA sequencing. The MARCKS cDNA consisted of human MARCKS, with a modified C-term to match the chick sequence EPAEPEPAE. Chick GAP43 (EDD) lacked amino acids 39-53; pMARCKS (ΔED) consisted of the human/chick protein, as described above, which lacked amino acids 152–176, and carried an additional Ala3Cys mutation to provide a palmitoylation sequence. A ntiser to COOH-terminal peptides of chick MARCKS and GAP43 were as described (Wiederkehr et al., 1997). Mouse MARCKS was detected with an antiserum to the COOH-terminal synthetic peptide SPEAPPAATAE; the mAb to PI(4,5)P₂ was as described (Fukami et al., 1998); RITC-phalloidin, RITC-α-bungarotoxin, and Alexa-labeled secondary antibodies were from Molecular Probes, Inc.

Cell lines (monkey kidney epithelial cells COS-7, and rat pheochromocytoma PC12 cells, clone B) were from American Type Cell Culture Collection, and cultured in DMEM supplemented with 10% FCS, or 10% horse serum and 5% FCS, respectively. Hippocampal neurons were isolated from newborn (P0-1) mice. In brief, hippocampi were triturated, cells were washed, resuspended in culture medium (neurobasal; GIBCO BRL), with 0.5 mM l-glutamate, 25 μM glutamate, and 1% B27 supplement (GIBCO BRL), and plated at a density of 20,000 cells per 18-mm poly-l-lysine-coated coverslip.

Transgenic mice expressing chick GAP43 (ΔED), specifically in adult neurons, were generated using the mouse Thy1.2 expression cassette as described (Caroni, 1997). Two independent lines of mice were analyzed in this study: line-13 expressed high levels of transgene (comparable to Thy1-GAP43(w2); Aigner et al., 1995) in most types of neurons, including spinal motorneurons, whereas line-19 exhibited widespread expression in the brain and motorneuron at levels that were at least three times lower than those of line-13, as judged by immunoblots (brain) and immunocytochemistry (neuromuscular junction).

Transfections, Immunocytochemistry, and Analysis of the Actin Cytoskeleton

Lipsome-based transfection reagents interfered with PI(4,5)P₂ stainings (not shown). Therefore, for transient transfections, COS-7 cells were treated with the nonliposomal reagent Superfect (Qiagen). On the next day, cells were fixed for 30 min at 37°C, followed by 3-5 h at 4°C with 4% paraformaldehyde in DME with 2 mM EGTA. Subsequently, cells were further processed for immunocytochemistry as described in a previous study (Wiederkehr et al., 1997). The first antibody binding incubation was carried out overnight at 4°C, using an antibody dilution solution consisting of PBS, with 0.2% saponin, 50 mM glycine, 0.1% BSA, and 1% FCS. Cultured hippocampal neurons from wild-type and transgenic mice were labeled 24 h after plating, according to the same protocol.

For quantitative analysis of GMC and PI(4,5)P₂ clusters, all cells from randomly selected fields (400 × 1) with substantial levels of transgene expression (30–50% of all transgene expressing cells in any given field) were included in the analysis. At least 20 cells from each clone of transiently transfected cells were analyzed for every experiment, and the values are averages from at least two independent experiments. Images from 20 μm² bins were captured and all clusters within the bin were analyzed with NIH Image software (see Fig. 2).

PC12 cells were transfected stably using the Fugene 6 reagent from Boehringer. Each experiment shown in the study was carried out with at least three independent clones, with similar results. For process outgrowth assays, 100,000 PC12 cells were plated on collagen-coated (30 μg/ml) 35-mm dishes, and, where indicated, the medium was changed 1 d after plating from DME, 10% horse serum, 5% FCS (growth medium) to DME, 1%...
hormone, and 100 ng/ml NGF. Where process formation (>1 cell diameter) in the absence of NGF was monitored, cells were preincubated with the growth factor for 2 h, replated in the presence or absence of the drug, and analyzed 3 h after replating. No preincubation was carried out when LiCl or the phospholipase C (PLC) inhibitor U-73122 was used.

To analyze the distribution of the actin cytoskeleton in PC12B clones, we determined intensity profiles of RITC-phalloidin labeling across randomly selected cells. Cells were plated on a collagen-coated substrate in the absence of NGF and stained 3 h after plating, photographed under identical conditions, and were analyzed with Image software. For each analyzed cell, one rectangular bin of 10 pixels in height was placed across the center of the cell (see also schematic in Fig. 6 B), and an edge-to-edge labeling intensity profile was collected. For each type of PC12B clone, such profiles had reproducible characteristic features of actin cytoskeleton distribution, as revealed when the profiles were superimposed. To highlight shared features, each of six independent profiles (i.e., six cells) was assigned the same light gray value, and overlapping areas were integrated (see Fig. 6 B).

Subcellular Fractionation and Lipid Analysis

Raf fractions from 2-d hippocampal neuron cultures or adult mouse brain homogenate were isolated according to a standard protocol (Maekawa et al., 1999). In brief, samples were processed with a Polytron homogenizer in hypotonic medium (10 mM Tris-HCl, 1 mM MgCl₂, 1 mM EGTA, with protease inhibitors), in the presence of 1% Triton X-100. The osmolality was adjusted to 0.8 M sucrose (total volume of 3 ml), and the homogenate was overlayed with 0.7 M (5 ml) and no sucrose (2 ml) Triton-containing solutions. An after centrifugation at 70,000 g for 6 h (4°C), the following three fractions were collected, sedimented in homogenization medium with 0.3% Triton X-100 (for 1 h at 100,000 g), and analyzed: (1) the fraction that had floated above 0.7 M sucrose (rafts); (2) 0.7–0.8 M sucrose interface, plus upper 1 ml of 0.8 M sucrose layer (0.7/0.8 fraction); and (3) the gradient pellet (high density membranes and Triton-insoluble cytoskeletal fractions). Raf fractions from PC12B clones were isolated by an analogous protocol, but cell lysates were prepared in the presence of 150 mM NaCl, 1% Triton X-100 and 0.1 M sodium carbonate, pH 11.

Binding of GMC proteins to phospholipids was determined with a sedimentation assay, as described previously (Seki et al., 1996). In brief, multilamellar phospholipid vesicles were made from phosphatidylcholine (Sigma Chemical Co.) or from a 1:9 mixture of PC and PIP mix (PI(4,5)P₂/PI(4)P/PI(3,4,5)P₃, containing 4.5 μg PC, 0.075 μg PIP and 0.075 μg PIP₃ lipids). Vesicles with bound proteins were sedimented at 150,000 g for 30 min and analyzed on immunoblots. Total masses of nonwater-soluble phosphoinositides were determined according to a standard protocol (Bird, 1998), using myo-2-[3H]-inositol (NEN) as a source of labeled inositol, PCA extraction, and TLC fractionation of phosphoinositides. The PI, PIP, and PIP2 spots were identified using phosphoinositide standards and iodine vapor. The appropriate spots were scraped off the TLC plates, extracted in ethanol/HCl (100:1), and counted in scintillation fluid. To compare PC12B clones, values were normalized to protein contents of acid-insoluble pellets. PIP, and PI(3,4,5)P₂ contents of NGF-treated PC12B clones were determined by [³²P]orthophosphate metabolic labeling and HPLC fractionation as described previously (Méier et al., 1998). PI(4)P levels were not affected by growth factor stimulation, and corresponding values were used to adjust individual sample counts per minute values for relative phosphoinositide recoveries.

Analysis of Peripheral Nerve Regeneration and BotA-induced Sprouting

To analyze peripheral nerve regeneration, the right sciatic nerve of 2-3-mo-old mice was crushed at the level of the midthigh, by applying pressure for 20 s with a watchmaker tweezer, as previously described (Aigner et al., 1995). Regeneration and reinervation of muscle was analyzed 14 d after the crush, using a combined silver esterase reaction (Carroni et al., 1997) on frozen sections of triceps surae muscle. For quantita-
GMC Bind PI(4,5)P₂ and Promote PI(4,5)P₂ Microdomain Assembly Independent of Actin Cytoskeleton Integrity

To determine whether GMC can influence PI(4,5)P₂-containing domains at the cell surface, we compared PI(4,5)P₂ labeling patterns in hippocampal neurons, PC12B cells, and COS cells expressing different levels of these proteins. As shown in Fig. 2 A, cells overexpressing GMC exhibited substantially larger macroscopic PI(4,5)P₂ clusters. In addition, MARCKS-overexpressing cells (but not those overexpressing GAP43 or CA P23; not shown) also exhibited stronger PI(4,5)P₂ cell-surface staining (Fig. 2 A). To exclude the possibility that the effects of GMC on the labeling pattern of PI(4,5)P₂ were related to the labeling process for these transgenes, we analyzed cells that had been transfected with a bicistronic construct driving the expression of the green fluorescent protein and MARCKS in the same transiently transfected cells. Cells expressing green fluorescent protein, and thus also MARCKS, exhibited larger, more numerous clusters and stronger labeling for PI(4,5)P₂ (not shown). Therefore, overexpression of GMC augments the formation of macroscopically detectable plasmalemmal PI(4,5)P₂ clusters.

To determine whether GMC proteins bind directly to PI(4,5)P₂, we carried out cosedimentation experiments with recombinant proteins and lipid vesicles. Binding of GMC to liposomes depended on the presence of acidic phospholipids such as PI(4,5)P₂ and on the presence of the basic ED (Fig. 2 B). Mutant GA P43(Ser42Asp), which does not bind calmodulin and codistributes with GMC at plasmalemmal microdomains (Wiederkehr et al., 1997), did bind to PI(4,5)P₂-containing vesicles, although less effectively than wild-type GA P43 (Fig. 2 B).

Cell-surface lipid microdomains described so far are highly sensitive to the physical properties of the lipid environment and, in particular, the presence of cholesterol (Harder and Simons, 1997). As shown in Fig. 2 C, treating COS cells with the cholesterol sequestering agent cyclodextrin, induced a loss of cluster and plasmalemmal PI(4,5)P₂. In transfected cells, this loss was accompanied by a concomitant reduction in the number and size of GMC clusters (Fig. 2 C). In addition, when compared with transgene-negative cells, overexpression of GA P43, MARCKS, or CA P23 partially protected cell surface and cluster PI(4,5)P₂ from cyclodextrin-induced dispersion (Fig. 2 C). In demonstrating that GMC-PI(4,5)P₂ cell-surface microdomains are highly interdependent, these results provide experimental evidence supporting the view that molecular interactions involving proteins such as GMC and phospholipids such as PI(4,5)P₂ are involved in the assembly and/or maintenance of these microdomains.

GA P43 and MARCKS can interact with actin filaments through their basic ED (Hartwig et al., 1995; He et al., 1997), and PI(4,5)P₂ interacts with a large number of actin-binding proteins (Welch et al., 1997). As a consequence, GMC may affect PI(4,5)P₂ microdomains through an indirect mechanism involving the actin cytoskeleton. To determine whether the association state of GMC-PI(4,5)P₂ microdomains depends on actin cytoskeleton integrity, we analyzed cluster size distributions in the presence of cytochalasin D. As shown in Fig. 3 A, treating COS cells acutely with 10 μM cytochalasin D for 20 min induced the loss of stress fibers and clumping of the actin cytoskeleton, but had no effects on PI(4,5)P₂ clusters, which remained significantly larger in the presence of MARCKS or GA P43. To investigate the effects of GMC on PI(4,5)P₂ microdomain assembly in the continued absence of an intact actin cytoskeleton, we analyzed COS cells cotransfected with GMC proteins and a constitutively active form of the small GTPase Hs1, which stimulates PI(3,4,5)P₃ generation and correlates with actin cytoskeleton assembly (Baj et al., 1996). Despite the presence of Hs1, transfection with GMC proteins was sufficient to disperse PI(4,5)P₂ clusters (Fig. 3 A).

Figure 1. GA P43, MARCKS, and CA P23 accumulate at plasmalemmal rafts, where they codistribute with PI(4,5)P₂. (A) Schematic representation of GMC, with the relative position of the EDs. (B) Plasmalemmal immunoreactivity detected with the PI(4,5)P₂ antibody k103 is suppressed by treatments that mask or reduce cell membrane PI(4,5)P₂. Treatment times are as follows: neomycin and LiCl, 24 h; and calcimycin, 15 min. (C) Codistribution of PI(4,5)P₂ immunoreactivity with endogenous GMC in hippocampal neuron growth cones and transiently transfected COS-7 cells. Note the absence of clustered labeling pattern for PI(4,5)P₂ and MARCKS in cells fixed with glutaraldehyde. (D) GMC accumulate in raft fractions from hippocampal neuron cultures, adult brain homogenate, and stably transfected PC12B clones. The samples are fractions from the sucrose density gradient used to isolate rafts; equal amounts of total protein were loaded on each lane. Lysate: brain homogenate. Bars: (B) 25 μm; (C) 3 μm.
Figure 2. The distributions of P(4,5)P₂ and GMC proteins at the cell membrane are interdependent. (A) Hippocampal neurons (left, nontransgenic; middle, transgenic overexpressing GAP43), COS cells (right) and PC12B cells overexpressing GMC proteins exhibit larger P(4,5)P₂ clusters. Quantitative analysis: COS cells, one 20 × 20 μm² bin analyzed per cell (n = 20). (B) GAP43 and MARCKS bind to P(4,5)P₂-containing PIPs but not to PC lipid vesicles; GAP43(Ser42Asp) binds more weakly; and GAP43(AED) does not bind. Quantitative analysis of GAP43 binding (immunoblot signal intensities, AU) to PIPs is shown in the graph (n = 4). (C) Cyclodextrin disperses plasmalemmal GMC-P(4,5)P₂ microdomains; GMC protect partially against dispersion by cyclodextrin. Raft fractions: PC12B-GAP43 cells, immunoblot. Photographs: COS cells treated with cyclodextrin (5 mM, 30 min), left; nontransfected (compare to nontreated patterns in A). Quantitative analysis: COS cells, with and without cyclodextrin; one 20 × 20 μm² bin per cell (n = 40). Bars: 10 μm.
of LIM-kinase1 ((LIM)K1) that phosphorylates cofilin and blocks actin cytoskeleton dynamics (Arber et al., 1998). As shown in Fig. 3 B, cells overexpressing the (LIM)K1 construct, with or without MARCKS (or GAP43, or CAP23; not shown) exhibited massive collapsing of the actin cytoskeleton into a few large clumps, but GMC-PI(4,5)P2 microdomains were not affected. Taken together, these results support the notion that GMC-PI(4,5)P2 microdomain assembly and modulation is independent of the actin cytoskeleton.

MARCKS and GAP43 Mutants Lacking the Basic Effector Domain Reduce Microdomain Size and Interfere with PI(4,5)P2 Retention at Plasmalemmal Clusters

GMC are acidic proteins, with one unique stretch of exclusively basic residues, that bind to acidic phospholipids like PI(4,5)P2 (Fig. 2 A). Because of its palmitoylation, GAP43 does not require the presence of the ED to efficiently associate with the plasma membrane (Wiederkehr et al., 1997). In contrast, PKC-mediated phosphorylation or deletion of the ED leads to the dissociation of MARCKS and CAP23 from the plasma membrane (Thelen et al., 1991; Aderem, 1995; Laux, T., and P. Caroni, unpublished observations). Consequently, to generate a MARCKS (ΔED) construct that still associated with the plasmalemma, we introduced an Ala3Cys point mutation to generate palmitoylated pMARCKS (ΔED). In spite of the absence of the lipid-binding ED in the mutants, in double-transfected cells overexpressing MARCKS or GAP43, and GAP43 (ΔED) or pMARCKS (ΔED), wild-type and mutant proteins codistributed at the cell surface (Fig. 4 A, bottom row). Therefore, accumulation of MARCKS or GAP43 at the characteristic surface-associated clusters, where these proteins colocalize, does not depend on the presence of the ED.

To explore the effects of the ED-free mutants on cluster assembly, we monitored their effects on PI(4,5)P2 clusters. Cells expressing comparatively low levels of pMARCKS (ΔED) or GAP43 (ΔED) exhibited cell-surface PI(4,5)P2 immunoreactivity that colocalized with the mutant transgene at small clusters (not shown). In contrast, hippocampal neuron growth cones and COS cells expressing substantial levels of GAP43 (ΔED) or pMARCKS (ΔED) exhibited transgene accumulation at numerous, regularly spaced, small plasmalemmal clusters, but were essentially devoid of plasmalemmal PI(4,5)P2 clusters (Fig. 4 A and B). These results suggest that the presence of excess ED-free mutant interferes with the function of endogenous components involved in the recruitment of PI(4,5)P2, and cluster coalescence. Since these mutants only differ from MARCKS or GAP43 by the absence of the ED that binds...
to acidic phospholipids, it seems likely that interference is due to a dominant negative mechanism, involving accumulation of the competing, nonfunctional component at these PI(4,5)P₂ sequestering platforms.

**PI(4,5)P₂ Microdomain Modulation by GMC Does Not Correlate with Alterations in Total Phosphoinositide Contents Nor Bradykinin-induced PI(4,5)P₂ Breakdown**

We next determined whether overexpression of GMC constructs alters lipid second messenger metabolism. First, we determined the total sizes of the PI, PIP, and PIP₂ pools in stably transfected PC12B clones (Bætge and Hammang, 1991) overexpressing comparable amounts of MARCKS, GAP43, or dominant negative pMARCKS (ΔED) transgenes (see also Fig. 6). These cells express low levels of endogenous MARCKS and CA P23 (not shown) and extremely low levels of endogenous GAP43 (Bætge and Hammang, 1991). GAP43- or pMARCKS(ΔED)-overexpressing cells did not exhibit alterations in bulk phosphoinositide levels (Fig. 5A). In contrast, overexpression of MARCKS produced a significant increase in phosphoinositide bulk levels in PC12B cells, a finding that is consis-

![Figure 4](https://example.com/figure4.png)
tent with the higher PI(4,5)P$_2$ immunoreactivity signals in cells transfected transiently with MARCKS. To determine whether overexpression of the GMC constructs affects signal-induced breakdown of PI(4,5)P$_2$, we analyzed bradykinin-induced PI(4,5)P$_2$ hydrolysis. This well characterized response to the activation of a G protein–coupled receptor involves the activation of PLC to hydrolyze PI(4,5)P$_2$ into inositol triphosphate and diacylglycerol. When compared with wild-type PC12B cells, the levels of stimulus-induced PI(4,5)P$_2$ metabolites were elevated in the presence of MARCKS, but not GAP43 (Fig. 5 B) nor pMARCKS (ΔED) (not shown). These results are consistent with the interpretation that MARCKS-overexpressing cells contain higher levels of the PLC substrate PI(4,5)P$_2$, whereas GAP43 or pMARCKS(ΔED) cells do not. Because GAP43 or pMARCKS(ΔED) overexpression did not alter bulk phosphoinositide levels nor stimulus-induced PI(4,5)P$_2$ breakdown, the results show that accumulation at PI(4,5)P$_2$-containing microdomains and augmentation or reduction of domain numbers and size does not, by itself, affect overall levels of PI(4,5)P$_2$ hydrolysis by activated PLC. As discussed below, the additional effects of MARCKS on bulk phosphoinositide levels may be due to the higher density of positive charges of its ED.

**Coordinate Regulation of PI(4,5)P$_2$ Microdomains and PI(4,5)P$_2$-sensitive Peripheral Actin Structures by GMC Constructs**

GMC proteins and PI(4,5)P$_2$ have both been implicated in regulating the accumulation of actin structures at the plasmalemma (Hartwig et al., 1992; Aigner and Caroni, 1995; Hartwig et al., 1995; He et al., 1997; Welch et al., 1997; Wiederkher et al., 1997). Modulation of microdomain PI(4,5)P$_2$ by GMC did not depend on the presence of an intact actin cytoskeleton (Fig. 3), which is consistent with the notion that it involves direct interactions between the EDs of GMC proteins and PI(4,5)P$_2$ at rafts. To explore the possibility that GMC modulation of plasmalemmal microdomain PI(4,5)P$_2$ may, in turn, regulate cell-surface actin dynamics, we analyzed the actin cytoskeleton of cells expressing GMC constructs. These experiments were carried out in neuronlike PC12 cells, where GAP43 promotes NGF-induced neurite outgrowth, a process that involves major rearrangements of the actin cytoskeleton (Paves et al., 1990). Three independent clones were analyzed for each construct, with comparable results. As shown in Fig. 6 and Fig. 7 A, overexpression of GAP43, GAP43(Ser42Asp), or MARCKS greatly potentiated the accumulation of actin-based filopodia and spikes at the periphery of untreated or NGF-treated PC12B cells. A comparison with wild-type PC12B clones revealed that, in the absence of NGF, this was accompanied by a dramatic decrease in cytosolic and perinuclear phalloidin signal (Fig. 6, A and B), suggesting the presence of a major shift towards the cell periphery in the subcellular accumulation of actin filaments. Indistinguishable results were obtained with PC12B clones overexpressing CAP23 (not shown). In marked contrast, GAP43(ΔED) or pMARCKS(ΔED) suppressed the accumulation of peripheral actin structures in response to NGF (Fig. 7 A). These findings are consistent with the view that PI(4,5)P$_2$ microdomain modulation by GMC promotes the assembly and accumulation of plasmalemmal actin-based structures.

To investigate more directly the possible involvement of PI(4,5)P$_2$ in GMC-regulated actin dynamics, we carried out experiments in the presence of 10 mM neomycin. This specific PI(4,5)P$_2$ sequestering agent (Fig. 1 B) had three major effects. First, it mimicked the overall effects of GMC on the distribution of the actin cytoskeleton, dramatically potentiating the accumulation of peripheral actin-based structures, particularly filopodia, at the plasmalemma, and greatly reducing the pool of comparatively amorphous cytosolic and perinuclear filamentous actin (Fig. 6, A and B, and Fig. 7 B). Second, it partially counteracted the effects of the dominant negative ΔED mutants (Fig. 7 B). Third, it counteracted the effects of GMC, with respect to the local accumulation of larger actin structures, cell polarization (Figs. 6 and 7 B), and neurite outgrowth (Fig. 8). As a result, cells treated with neomycin spread in radially symmetrical, round shapes, with dense accumulations of evenly distributed actin-based filopodia and lamellae at their edges. The effects of neomycin were mimicked most closely by the GA P43(Ser42A sp) mutant that does not bind calmodulin (CaM) and cannot be phosphorylated by PKC (Figs. 6 B and 7 A), suggesting that regulation of GMC proteins by calcium/CaM and PKC may promote the local accumulation of larger actin structures (see Fig. 10). The neomycin effects did not depend on the presence of NGF, which, like GMC proteins, potentiated the accumulation of larger peripheral actin structures, particularly spikes (Fig. 6 A). Interestingly, GMC and NGF had similar effects on neomycin-treated PC12 cells, and their combination partially rescued actin accu-
Figure 6. Roles of GMC and PI(4,5)P₂ in actin regulation at the cell periphery. (A) RITC-phalloidin patterns of PC12B cells 3 h after plating. The figure shows representative examples obtained with a clone expressing MARCKS; comparable results were obtained with GAP43 and CAP23 clones. (B) Quantitative analysis of phalloidin labeling profiles (see Materials and Methods for details). The schematic on the left shows how rectangular bins were placed (six cell profiles are superimposed for each plot on the right). Bar, 25 μm.
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Figure 7. Opposite effects of GMC and their ΔED mutants on plasmalemmal actin dynamics. RITC-phalloidin labelings of PC12B clones. (A) Cells grown in the presence of NGF for 4 d. Rectangles show the position of the details shown in the bottom rows. (B) Cells grown for 1 d in the presence of NGF and drugs, as indicated. Note the induction of symmetrical actin structures in the presence of neomycin, partial rescue of polarization by MARCKS, and opposite effects of the PLC inhibitor U-73122. Also note that neomycin partially rescued peripheral actin accumulation in GAP43(ΔED) cells. Bar: 10 μm (A, top rows); 25 μm (B).

Critical Role of Plasmalemmal GMC for Neurite Outgrowth in PC12 Cells

To investigate possible biological implications of microdomain and actin regulation by GMC proteins, we analyzed NGF-induced neurite outgrowth in stable PC12B clones overexpressing wild-type and ED-deficient con-
structs. As expected (Yankner et al., 1990), overexpression of GAP43 substantially potentiated cell spreading and neurite outgrowth (Fig. 8 A). MARCKS- or CAP23-overexpressing cells exhibited potentiated neurite outgrowth indistinguishable from that induced by GAP43 (Fig. 8 A). This is remarkable, given the absence of sequence homologies between the three proteins, and is consistent with their similar effects on PI(4,5)P2-containing microdomains and the actin cytoskeleton. Dominant negative GAP43(ΔED) or pMARCKS(ΔED) suppressed NGF-induced neurite outgrowth (Fig. 8 A). These findings are entirely consistent with the effects of GMC constructs on PI(4,5)P2-containing microdomains and actin dynamics, and provide evidence for a critical role of GMC protein-mediated regulation in neurite outgrowth.

To determine whether GMC can induce process outgrowth in these cells in the absence of NGF, we monitored attachment and spreading of PC12 clones on a collagen substrate. Unlike wild-type cells, GMC-overexpressing cells formed neurite-like processes during spreading in the presence of high serum concentrations and no NGF (Fig. 8 B). Under these experimental conditions, process formation was transient: it peaked at ~3 h after plating, and processes disappeared after 15 h (not shown). In contrast to those formed in the presence of NGF, processes were short, not exceeding 50 μm in length, and were not affected by the presence of the PI3-kinase inhibitor LY294002 (not shown). The specific PI(4,5)P2 sequestering agent neomycin promoted symmetrical cell spreading, and prevented spontaneous process formation (Fig. 8 B) and NGF-induced neurite outgrowth (not shown, but see Fig. 7 B) in GMC-overexpressing cells. Comparable effects were obtained with 10 mM LiCl (Fig. 8 B), which inhibits the metabolism of phosphoinositides, and greatly reduces the levels of plasmalemmal PI(4,5)P2 (Fig. 1 B). In contrast to neomycin, U-73122, which directly inhibits PLC, effectively inhibited cell spreading (see also Fig. 6 A).

NGF-induced neurite outgrowth involves the activity of PI3-kinases (Jackson et al., 1996), which may depend, in part, on the local concentrations of plasmalemmal substrate phosphoinositides available for phosphorylation. However, neither GMC proteins nor their ΔED mutants affected the accumulation of the 3-phosphoinositides PIP3 (Fig. 8 D) or PI(3,4)P2 (not shown) in response to NGF, which is consistent with the notion that the regulation of neurite outgrowth by GMC proteins specifically involves PI(4,5)P2.
Role of GMC-mediated Regulation in Peripheral Nerve Regeneration and Stimulus-induced Nerve Sprouting at the Neuromuscular Junction in the Adult

Although GAP43 has been implicated in axonal growth, GAP43-deficient mice only exhibit restricted axonal pathfinding defects (Strittmatter et al., 1995; Sretavan and Kruger, 1998). Likewise, MARCKS-deficient mice do form axonal projections, although these are highly aberrant (Stumpo et al., 1995). Our results (Frey et al., 2000a) provide strong evidence that these proteins have partially redundant functions, suggesting that to reveal their full functional roles would require approaches that interfere with common downstream components. Therefore, we generated transgenic mice overexpressing dominant negative GAP43 (ΔED) specifically in neurons. We used a mouse Thy1.2 expression cassette, thus, achieving high expression levels restricted to postnatal neurons, including motoneurons and their neuromuscular synapses (Fig. 9 A; Caroni, 1997). Hippocampal neurons derived from such mice exhibited a disruption of growth cone plasmalemmal PI(4,5)P₂ clusters.
The circular course was reminiscent of CAP23 frequently exhibited a strikingly circular course (Fig. 9 B). In addition, the nerve frequently exhibited a strikingly circular course (Fig. 9 B). The circular course was reminiscent of CA P23-/− neurons, where the phenotype could be phenocopied by cytochalasin D, supporting the notion that it was due to a defect in the actin cytoskeleton (Frey et al., 2000a).

GA P43 and CA P23 promote nerve sprouting at the neuromuscular junction (Agner et al., 1995; Caroni et al., 1997; Frey et al., 2000a). To determine the effect of GA P43(ΔED) on stimulus-induced nerve sprouting, we paralyzed lower hindlimb muscles of transgenic mice with botulinum toxin A. 7 d after toxin treatment, >95% of the synapses in the soleus muscle of wild-type mice exhibit robust ultraterminal sprouting, whereas sprouting in the medial gastrocnemius is much less pronounced, and is restricted to slow-type synapses (Frey et al., 2000b; Fig. 9, C and D). As predicted, sprouting in the soleus muscle of transgenic mice was greatly reduced in frequency and length (Fig. 9, C and D). However, in addition, a large proportion of medial gastrocnemius synapses that do not sprout in wild-type mice, nor in Thy1-GA P43 mice (Frey et al., 2000b), did so in the presence of GA P43(ΔED) (Fig. 9 D). Sprouting in the absence of toxin treatment was minimal (Fig. 9 D), suggesting that the presence of GA P43(ΔED) interfered with negative control of stimulus-induced sprouting at these synapses. When compared with those induced in wild-type mice, sprouts were strikingly curved, with frequent side branches and local expansions (Fig. 9 C). A gain, these features were reminiscent of the abnormal neurite outgrowth patterns in CA P23-/− or cytochalasin D–treated neurons (Frey et al., 2000a), which is consistent with the notion that they may reflect a disruption in actin cytoskeleton control at the cell cortex.

Finally, to explore the role of GM C proteins in axonal regeneration, we crushed the sciatic nerve of Thy1-GA P43 (ΔED) mice and monitored peripheral nerve regeneration. A shown in Fig. 9 (E, F), GA P43(ΔED) inhibited regeneration and reinnervation of skeletal muscle in a dose-dependent manner. U ltrastructural analysis of myelinated axonal profiles proximal to the crush site revealed normal numbers in the mutant mice (not shown). In addition, although with a substantial delay, skeletal muscle was apparently fully reinnervated also in the transgenic line expressing high levels of GA P43(ΔED). Therefore, the presence of excess GA P43(ΔED) greatly delayed, but did not completely prevent peripheral nerve regeneration. These results are consistent with the in vitro data with PC12 cells, and indicate that regulation by GM C proteins plays a critical role in axonal outgrowth in vivo.

Discussion

We have provided evidence that GA P43, MARCKS, and CA P23 regulate plasmalemmal microdomain PI(4,5)P2, cell cortex actin recruitment, and morphogenetic processes such as neurite outgrowth. The effects of GM C constructs on the actin cytoskeleton and morphogenetic processes correlated closely with those on plasmalemmal PI(4,5)P2, and were mimicked by pharmacological agents that act on PI(4,5)P2, which is consistent with the notion that a main function of these proteins is to locally modulate PI(4,5)P2, upstream of actin cytoskeleton and cell cortex regulation. In the following sections, we discuss the regulation of PI(4,5)P2 microdomains by GM C-like proteins and their function in actin dynamics, neurite outgrowth, and anatomical plasticity.

A Lipid Microdomain that Brings Together PI(4,5)P2 and the Plasmalemma-associated Proteins GAP43, MARCKS, and CAP23

The results of this study suggest that PI(4,5)P2 and GM C proteins accumulate together at a subtype of cholesterol-rich plasmalemmal microdomains found in many, and possibly all types of cells. Thus, endogenous and transgenic G M C accumulated in cyclodextrin-sensitive raft fractions (Fig. 1 D; Maekawa et al., 1999), where they codistributed with PI(4,5)P2 (Liu et al., 1998; Pike and Miller, 1998) in a surface-associated patchy pattern in all cell lines and primary cell types that we have tested. This characteristic pattern did not overlap with the distribution of a number of surface-associated components, including early endosomes, caveolin, integrin-β1, and vinculin, which also yield patchy labeling patterns. The microdomains described in this study appear to share significant physico-chemical properties with sphingolipid/GPl-linked protein microdomains (Barde and Simons, 1997). However, the absence of substantial colocalization between GM C-PI(4,5)P2 and src or caveolin (Wiederkehr et al., 1997) suggest that these microdomains may not overlap physically with sphingolipid/GPI-linked protein rafts, and that GM C-PI(4,5)P2-containing microdomains may belong to a subtype of rafts.

Definitive information about the properties and the regulation of GM C-PI(4,5)P2 microdomains will require further studies, including their biochemical isolation, and molecular identification of their lipid and protein components, as well as a characterization of their dynamic properties. However, some conclusions can already be drawn from this study. First, like previously described rafts, they are sensitive to cholesterol-depleting drugs, implying that significant domain promoting forces possibly related to those operating in sphingolipid rafts reside within the lipid environment. Second, direct interactions between PI(4,5)P2 and GM C proteins appear to promote PI(4,5)P2 retention at the domains. Thus, overexpression of GM C, induced larger macrodomains, and partially counteracted cyclodextrin-induced loss and dispersion of plasmalemmal PI(4,5)P2. In contrast, effector domain mutant accumulation may reduce the net binding capacity of the domain for PI(4,5)P2, thus, reducing masking and retention of this lipid second messenger. A long similar lines, the higher total contents of PI(4,5)P2 in MARCKS, but not in GA P43-overexpressing cells may be due to the fact that the E D of MARCKS has a significantly higher density of basic residues (13 lysine and arginine out of a total of 25 residues in
M A R C K S, versus 8/23 in G A P 43), thus, binding PI(4,5)P₂ with higher avidity. These combined findings suggest that direct electrostatic interactions between the E D-containing proteins and acidic phospholipids, including PI(4,5)P₂, may be involved in domain stabilization. However, additional, presently unidentified, components are likely to be involved in microdomain nucleation and recruitment. Thus, for example, since mutant constructs of M A R C K S or G A P 43 lacking the E D codistribute with the wild-type proteins and PI(4,5)P₂, protein interactions not involving the E D must be involved in G M C targeting to these domains.

With respect to their regulation, an important difference to previously described rafts is that while sphingolipid/G P I-linked protein complexes occupy the outer leaflet of the plasmalemma, where they can be regulated by signals from the extracellular environment, G M C–PI(4,5)P₂ complexes are located at the inner leaflet. The interaction of the basic domains of G M C with acidic phospholipids, including PI(4,5)P₂, is subject to regulation by several signaling pathways (A derem, 1995; B enowitz and R ottenberg, 1997). A coordinately, phosphorylation by P K C or binding of calcium/C a M would mimic E D deletion by masking positively charged E Ds, thereby weakening electrostatic interactions that sequester PI(4,5)P₂. As a consequence, G M C proteins may mask and inhibit microdomain PI(4,5)P₂ under resting conditions, and release clustered PI(4,5)P₂ in response to local calcium/C a M and/or P K C activation (Fig. 10).

Functions of G M C Proteins in Actin Regulation, Neurite Outgrowth, and Anatomical Plasticity

The question of whether G M C are signaling or structural proteins, and what, if any, are their downstream targets has been difficult to address experimentally in the past. Because they can be such abundant proteins, and because their E Ds interact with several molecules involved in signaling, including calcium/C a M, P K C, PI(4,5)P₂, and actin filaments, it has been suggested that M A R C K S and G A P 43 may regulate the pools of any of these interacting molecules. However, we now find that G M C can modulate PI(4,5)P₂ independent of binding to C a M or actin cytoskeleton integrity, and that all effects of G M C on the actin cytoskeleton, cell spreading and neurite outgrowth correlate with their effects on microdomain PI(4,5)P₂. The global effects on cell spreading and peripheral actin recruitment are mimicked by drugs that reduce the availability of PI(4,5)P₂, whereas the effects on cell polarization and process formation are, in part, prevented by these same drugs.

This may reflect the regulatory cycle outlined above, i.e., masking of PI(4,5)P₂ under resting conditions, which is mimicked by neomycin, and local unmasking of clustered PI(4,5)P₂ in response to calcium/C a M and/or P K C activation, which is not mimicked by neomycin (Fig. 10). This interpretation is also consistent with the observation that G A P 43(Ser42A sp), which does not bind C a M, cannot be regulated by P K C, but still binds PI(4,5)P₂, affected the actin cytoskeleton in a manner that was particularly reminiscent of that of neomycin (Figs. 6 B and 7 A). Therefore, we propose that PI(4,5)P₂ is a main effector of G M C, and that, rather than being regulated by G M C, calcium/C a M and P K C regulate the interactions of G M C with PI(4,5)P₂. A coordinately, a main function of G M C would be to act as PI(4,5)P₂ modulatory pimodulins, to retain and mask PI(4,5)P₂ at plasmalemmal lipid microdomain platforms, where they would couple its availability for actin cytoskeleton and cell cortex regulation to signal transduction pathways involving calcium/C a M and P K C (Fig. 10). In addition to modulating PI(4,5)P₂, M A R C K S and G A P 43 can also interact directly with actin filaments (H artwig et al., 1992; H e et al., 1997). As a consequence, in spite of the striking correlations between the effects of G M C and those of pharmacological agents that act on PI(4,5)P₂, some of the effects described in this study may be due to such direct interactions. The observation that the colocalization between G M C proteins and actin filaments is very limited (Wiederkehr et al., 1997) seems to argue against this possibility. In addition, under cell-free conditions, where filament decoration by other proteins was readily detectable by electron microscopy, we could not detect any decoration of actin monomers or filaments by recombinant G A P 43 or C A P 23 (C a roni, P., unpublished results). Therefore, although we presently cannot exclude more direct roles in actin dynamics regulation, we favor the possibility that interactions between G M C proteins and actin filaments may synergize with modulation of PI(4,5)P₂ to facilitate the recruitment and assembly of actin-based structures.

How does G M C-modulated PI(4,5)P₂ affect the actin cytoskeleton and cell-surface activity? Based on the results of this study, the fact that G M C expression correlates with cell cortex dynamics, and the known effects of PI(4,5)P₂ on the actin cytoskeleton and the cell cortex, we suggest that G M C levels affect local PI(4,5)P₂ availability, that in turn directly controls the activity of key actin regulating proteins. This model is consistent with recent evidence that PI(4,5)P₂ promotes membrane-cortical cytoskeleton interactions (R aucher et al., 2000), and that...
plasmalemmal actin polymerization promotes cell spreading (Vasioukhin et al., 2000). According to in vitro system studies, sequestration of PI(4,5)P₂ should consistently promote cell-surface actin dynamics, facilitating stimulus-induced actin recruitment, whereas its local liberation should promote growth and stabilization of peripheral actin structures (for reviews see Welch et al., 1997; Ma et al., 1998; Rac1er et al., 2000). First, inhibition of gelsolin by PI(4,5)P₂ should reduce actin severing and dynamics (e.g., Hartwig et al., 1995). Second, global inhibition of coflin by PI(4,5)P₂ should reduce monomer dissociation at the minus end of filaments, thus, reducing actin filament dynamics (Welch et al., 1997). Third, sequestration of profillin by PI(4,5)P₂ should globally inhibit filament growth, but promote local growth by uncapping filament plus ends (Hartwig et al., 1995). In addition, binding of WASP proteins to PI(4,5)P₂ promotes Arp2/3 function and actin recruitment, and binding of PI(4,5)P₂ to vinculin should promote focal contact formation.

The PI(4,5)P₂ modulatory mechanism suggested by our results is likely to be operating throughout the cell surface. Thus, although some local accumulation can be detected at sites of cell-substratum attachment and ruffling activity, the immunocytochemistry data suggest the presence of substantial amounts of evenly distributed PI(4,5)P₂ and GMC throughout the cell cortex. However, because it should be affected by the intrinsic, cell-specific properties of the cortical cytoskeleton, and by the local accumulation of structural, regulatory, and signaling components that initiate and regulate actin structure formation, its outcome is likely to exhibit highly cell specific and local features. Taking all these factors into account, actin regulation by GMC and PI(4,5)P₂ may involve the following mechanisms (see also Fig. 10). First, under local resting conditions, with low contents of components such as Arp2/3, CapZ, and activated Rho-type GTPases that promote the formation of dynamic structures, and with low levels of GMC proteins, accessible PI(4,5)P₂ would favor cortical actin cytoskeleton stability, suppressing local dynamics and inhibiting membrane fusion. Second, under the same conditions, but with higher levels of GMC proteins, PI(4,5)P₂ masking would lower the threshold for cell-surface dynamics, promoting processes such as cell spreading and membrane fusion. Third, in the presence of signals and components that promote the formation of actin structures, for example, at a forming axonal growth cone or phagosome, stimulus-induced release of PI(4,5)P₂ from masking by GMC would support local actin filament assembly, coupling this process to regulation by calcium/CaM and PKC signals. Overall, according to this model, the expression of proteins such as GAP43, MARCKS, or CapZ, and activated Rho-type GTPases that promote and regulate cell-surface dynamics, phagocytosis, cell attachment, and regulated morphogenic processes such as neurite outgrowth (Fig. 10). These predictions are in good agreement with a wealth of data from cultured cells and genetically modified mice.

The effects of GMC constructs on neurite outgrowth in PC12 cells, peripheral nerve regeneration, and stimulus-induced nerve sprouting at the neuromuscular junction of soleus muscle provide strong experimental evidence for a critical role of GMC proteins in promoting process outgrowth. Together with those of the accompanying paper (Frey et al. 2000a), they establish GMC proteins as major intrinsic determinants of anatomical plasticity in neurons. In addition, the results highlight the role of cell cortex regulation in controlling anatomical plasticity. Thus, overexpressing GAP43(ΔED) in neurons not only impaired proper axonal regeneration and synaptic sprouting, but also paradoxically allowed paralysis-induced sprouting at neuromuscular synapses that do not sprout in wild-type mice. Such sprouts were abnormal in shape and exhibited several features reminiscent of cytochalasin D–treated or CapZ3/– neurons. It is well established that disrupting the actin cytoskeleton of pioneer neurons in situ abolishes growth cone guidance and induces extensive twisted growth of neurites (Bentley and Toriohan-Raymond, 1986). In a similar manner, intrinsic determination of anatomical plasticity in neurons may involve differential expression of components that control the dynamics of the actin cytoskeleton at the cell cortex, and interference with this control would disrupt specificity in stimulus-induced anatomical plasticity.

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