Phosphorylation-Site Mutagenesis of the Growth-associated Protein GAP-43 Modulates Its Effects on Cell Spreading and Morphology

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Abstract. The 43-kD growth-associated protein (GAP-43) is a major protein kinase C (PKC) substrate of axonal growth cones, developing nerve terminals, regenerating axons, and adult central nervous system areas associated with plasticity. It is a cytosolic protein associated with the cortical cytoskeleton and the plasmalemma. Membrane association of GAP-43 is mediated by palmitoylation at Cys_3Cys_4. In vitro and in vivo, phosphorylation by PKC exclusively involves Ser_{41} of mammalian GAP-43 (corresponding to Ser_{42} in the chick protein).

To identify aspects of GAP-43 function, we analyzed the actions of wild-type, membrane-association, and phosphorylation-site mutants of GAP-43 in nonneuronal cell lines. The GAP-43 constructs were introduced in L6 and COS-7 cells by transient transfection. Like the endogenous protein in neurons and their growth cones, GAP-43 in nonneuronal cells associated with the cell periphery. GAP-43 accumulated in the pseudopods of spreading cells and appeared to interact with cortical actin–containing filaments. Spreading L6 cells expressing high levels of recombinant protein displayed

a characteristic F-actin labeling pattern consisting of prominent radial arrays of peripheral actin filaments. GAP-43 had dramatic effects on local surface morphology. Characteristic features of GAP-43–expressing cells were irregular cell outlines with prominent and numerous filopodia. The effects of GAP-43 on cell morphology required association with the cell membrane, since GAP-43(Ala_3Ala_4), a mutant that failed to associate with the cell cortex, had no morphogenetic activity. Two GAP-43 phosphorylation mutants (Ser_{42} to Ala_{42} preventing and Ser_{42} to Asp_{42} mimicking phosphorylation by PKC) modulated the effects of GAP-43 in opposite ways. Cells expressing GAP-43(Asp_{42}) spread extensively and displayed large and irregular membranous extensions with little filopodia, whereas GAP-43(Ala_{42}) produced small, poorly spreading cells with numerous short filopodia.

Therefore, GAP-43 influences cell surface behavior and phosphorylation modulates its activity. The presence of GAP-43 in growing axons and developing nerve termini may affect the behavior of their actin-containing cortical cytoskeleton in a regulatable manner.

T HE 43-kD growth-associated protein (GAP-43),[1] a unique protein of the nervous system, has attracted much attention because its properties suggest that it is involved in neuronal development and plasticity. Its presence in neurons correlates with axonal growth during development and regeneration (Skene and Willard, 1981; Basi et al., 1987; Skene, 1989; Coggins and Zwiers, 1991) and possibly with plasticity during the development of synaptic connections and in the adult central nervous system (Jacobson et al., 1986; Benowitz and Routtenberg, 1987). It is also a major protein kinase C (PKC) substrate of growth cones and nerve termini (Skene et al., 1986; Meiri et al., 1986; Nelson et al., 1989); and changes in its state of phosphorylation correlate with plasticity (Benowitz and Routtenberg, 1987).

GAP-43 is a highly hydrophilic cytosolic protein that is anchored to membranes shortly after its biosynthesis via fatty acylation at its NH_2-terminal end (Cys_3Cys_4; Skene and Virág, 1989; Zuber et al., 1990; Liu et al., 1991). This modification may be dynamic, thus generating two cellular pools of the protein (Skene and Virág, 1989). Efficient attachment to membranes after biosynthesis is probably responsible for its rapid transport down the axon and subsequent accumulation at the growth cone or nerve terminus. Additional sequences on GAP-43 may be responsible for its preferential accumulation into growing axons of cultured hippocampal and embryonic neurons (Goslin et al., 1988; Liu et al., 1991). In growth cones, nerve termini, and axons, a large proportion of GAP-43 is tightly associated with the actin-rich cortical cytoskeleton (Allsopp and Moss, 1989; Meiri and Gordon-Weeks, 1990; Moss et al., 1990). Whether GAP-43 specifically binds to cortical cytoskeleton proteins is presently not known.

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1. *Abbreviations used in this paper:* GAP-43, 43-kD growth-associated protein; PKC, protein kinase C.

In situ, the PKC-mediated phosphorylation of GAP-43 exclusively involves a single site (Ser_{41} in mammals, corresponding to Ser_{42} in chick and goldfish GAP-43; Coggins and Zwiers, 1991; Meiri et al., 1991). Additional phosphorylation sites at Ser_{96} and Thr_{172} were recently reported (Spencer et al., 1992), but the kinase responsible for these phosphorylation reactions has not been identified. PKC-mediated phosphorylation of GAP-43 probably takes place in the growth cone and at the nerve terminus, since a mAb that specifically binds to phosphorylated (Ser_{41}) GAP-43 selectively detects the distal-most portion of growing peripheral axons (Meiri et al., 1991). Neurite outgrowth on laminin and on a variety of matrix and cell surface proteins is mediated by the activation of PKC (Bixby, 1989). In addition, nerve growth factor induces the phosphorylation of growth cone particle GAP-43 (Meiri and Burdich, 1991). Therefore, PKC-mediated phosphorylation of growth cone GAP-43 may be an important downstream response during axonal growth. Likewise, GAP-43 phosphorylation may be an important mediator of PKC activation in developing nerve termini and in some areas of the adult central nervous system that have been associated with plasticity.

In spite of extensive studies, the biological function of GAP-43 is not known. The recent demonstration that the absence of GAP-43 in a mutant PC12 cell line did not affect its ability to extend neurites clearly indicates that the presence of GAP-43 is not a prerequisite for process extension (Baetge and Hammang, 1991; but see Shea et al., 1991). On the other hand, in cell-free systems, GAP-43 interacts with a number of proteins involved in intracellular signaling, including calmodulin (Cimler et al., 1987) and Go (Strittmatter et al., 1990); and antibodies to GAP-43 have been reported to inhibit evoked secretion in permeabilized synaptosomes (Dekker et al., 1989) and neurite outgrowth in neuroblastoma cells (Shea et al., 1991). In addition, it was reported that the expression of GAP-43 in nonneuronal cells promotes the formation of large filopodia during cell attachment to the substrate (Zuber et al., 1989). Filopodia formation was a transient phenomenon, but these experiments clearly indicated that GAP-43 can affect surface behavior.

We have now investigated aspects of GAP-43 function by expressing the recombinant protein in cell lines. These consistently responded to increasing levels of GAP-43 with major cell surface reactions, including the formation of filopodia and process-like extensions. We find that GAP-43 accumulates in pseudopodia and other actin-rich cortical structures, where it appears to interact with cortical actin-containing filaments; and, furthermore, that its morphogenetic activity depends on its association to the cell surface. Using site-directed mutagenesis, we generated two phosphorylation-site mutants of chick GAP-43. In one mutant, Ser_{42} was replaced by an alanine to prevent phosphorylation. The second mutant was based on the finding that substitution of a serine or a threonine residue by a negatively charged amino acid (glutamate or aspartate) is an effective way to mimic a constitutively phosphorylated protein (Thorness and Koshland, 1987; Ducommun et al., 1991; Tavarè et al., 1991). Using the different constructs, we show that phosphorylation and mutation of Ser_{42} in GAP-43 modulate its effects on cell morphology. Our findings indicate that GAP-43 modifies local cell cortex behavior in a PKC-sensitive manner, suggesting that its presence in growth

cones and nerve terminals may affect the dynamic behavior of their actin-containing cortical cytoskeleton in a regulatable manner.

Materials and Methods

Reagents and Cells

Cytochalasin B (stock solution: 5 mM in DMSO) and PMA (stock solution: 10 μM in DMSO) were from Sigma Chemical Co. (St. Louis, MO). RITC-phalloidin (stock solution: 200 U/ml in methanol), biotin anti-mouse-, biotin anti-rabbit-, and Lucifer yellow-conjugated streptavidin were from Molecular Probes Inc. (Eugene, OR). Oligonucleotides were synthesized using a DNA synthesizer (model 308B; Applied Biosystems, Inc., Foster City, CA). The mAb 5F10 specifically detected chick GAP-43 (Widmer and Caroni, 1990). Rabbit antiserum to peptide GLVVMNIT (internal sequence of the extracellular matrix protein tenascin), which detected this peptide only when it was located at the COOH-terminal end of proteins, was a gift of Dr. R. Chiquet-Ehrismann (Friedrich Miescher Institute, Basel, Switzerland).

Cell lines were cultivated on polylysine (Sigma Chemical Co.)-coated substratum in DME with 10% FCS. When indicated, cells were detached from the substratum in the presence of 0.25% trypsin, washed in culture medium, and replated at an approximate density of 10^5 cells/cm^2. Rat myogenic L6 and monkey kidney epithelial COS-7 cells were from American Type Culture Collection (Rockville, MD). Embryonic 8-d chick dorsal root ganglia explants were cultivated in the presence of nerve growth factor on a laminin-coated substratum for 1 d before fixation and labeling.

Nucleic Acids and Transfection Experiments

cDNA coding for chick GAP-43 was isolated using polymerase chain reaction technology with the primers $^-12$ to $^+12$ (sense) and $^+741$ to $^+765$ (antisense) as deduced from the sequence of Baizer et al. (1990). To verify that the sequence was correct, complete sequencing was performed (chain termination method; Sequenase II kit; United States Biochemical Corp., Cleveland, OH). The complete coding sequence of chick GAP-43 was then inserted either in the eukaryotic expression vector pECE (SV40 promoter; Ellis et al., 1986) or in an M13 vector (Sambrook et al., 1989). Mutations in chick GAP-43 cDNA were introduced by a standard primer extension protocol using the M13 vector (Kunkel et al., 1987) and complete sequences of the mutants were derived. The extension GLVVMNIT was added to the COOH-terminal end of chick GAP-43 by applying appropriate polymerase chain reaction technology, starting from the corresponding cDNA. For subsequent expression, mutated cDNAs were introduced into the vector pECE, as described above. For control experiments, the original pECE vector was used in mock transfection experiments and the coding sequence of chick CAP-23 (Widmer and Caroni, 1990) was inserted in pECE as described above.

Plasmid DNA was transfected into eukaryotic cell lines with the calcium-phosphate precipitation method (Chen and Okayama, 1987). Cells were exposed to a glycerol shock 16 h after transfection, fixed 48 h after transfection, and processed for immunocytochemistry as described below. Where indicated, cells were detached 44 h after transfection, replated on polylysine-coated coverslips, and processed for immunocytochemistry 4 h later. Transfection efficiencies ranged from 5 to 30%; ~40% of the expressing cells displayed signals comparable to those shown in Fig. 1 a.

Immunocytochemistry

Chick GAP-43 was visualized with the specific mAb 5F10 (1:1,000 dilution of ascitic fluid; Widmer and Caroni, 1990). Chick CAP-23 was detected with the specific mAb 15C1 (Widmer and Caroni, 1990). In control experiments, the COOH-terminal sequence GLVVMNIT was detected with a specific rabbit antiserum (1:500 dilution). Cells were either fixed for 30 min in PBS with 4% paraformaldehyde at 37°C and permeabilized for 10 min in PBS with 0.1% NP-40 and 5% BSA or fixed for 3 min in methanol at −70°C and then incubated for 10 min in PBS with 5% BSA. Further incubations were at room temperature in PBS with 1% BSA. Cells were washed in PBS after each incubation with antibody. Concentrations of biotinylated second antibodies were 5 μg/ml. To visualize bound biotinylated antibody, cells were finally incubated in the presence of 3.5 μg/ml of Lucifer yel-

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low-conjugated streptavidin. When indicated, RITC-conjugated phalloidin (1 U/ml) was included in the final incubation mixture. Finally, cultures were extensively washed, mounted in Gelvatol (Monsanto Co., St. Louis, MO); fluorescence was detected with a microscope (Axiovert-10; Carl Zeiss, Inc., Thornwood, NY) equipped with the appropriate filters (Lucifer yellow: excitation 395–440 nm, emission 460 nm; RITC: excitation 510–560 nm, emission 580 nm). Control experiments with mock-transfected cells and appropriate nonreactive antibodies were performed to verify the specificity of the signals.

To estimate spreading areas of transgene-expressing and phalloidin-labeled cells, randomly selected low-magnification fields were analyzed with Image 1.4 (National Institutes of Health, Bethesda, MD) software.

Results

The coding sequence of chick GAP-43 (Baizer et al., 1990) was expressed transiently in the myogenic rat cell line L6 and in COS-7 cells. Transient expression was used to monitor dose-dependent effects of GAP-43 on cell morphology; recombinant protein was detected by immunocytochemistry with a mAb that specifically detects chick GAP-43 (Widmer and Caroni, 1990). Recombinant protein was detected in a characteristic surface-associated pattern that was distinctly punctate when cells were fixed in the presence of paraformaldehyde and less coarsely punctate upon rapid fixation in cold methanol. In control experiments, identical labeling patterns were observed when the peptide sequence GLV-VMNIT was added to the COOH-terminal end of GAP-43 and the fusion protein was detected with a peptide-specific antiserum, indicating that the mAb used in this study did not react preferentially with a subpopulation of fixed GAP-43 (data not shown). As shown in Fig. 1, recombinant GAP-43 in L6 cells (Fig. 1, a and b) and endogenous GAP-43 in neuronal growth cones (Fig. 1 c) yielded comparable labeling patterns, indicating that GAP-43 associated with similar cellular structures in neurons and nonneuronal cells.

Expression of GAP-43 in Myogenic L6 Cells Alters Cell Morphology

2 d after transfection, L6 cells expressing high levels of GAP-43 displayed extensive local surface irregularities, including filopodia, process-like extensions, and incomplete retraction figures (Fig. 1 a). Signal levels for GAP-43 in these cells were comparable to those of endogenous GAP-43 in neuronal growth cones (Fig. 1 c). Cells expressing low levels of GAP-43 had more regular outlines and were not decorated by filopodia (Fig. 1 b). The cell shown in Fig. 1 a is part of a confluent monolayer 2 d after transfection; its morphology is representative of most L6 cells that expressed high levels of GAP-43. However, spreading cells 3 h after replating were compared in most experiments shown here.

To determine whether the expression of GAP-43 affected the morphology of L6 cells, expressing and nonexpressing cells were compared in the transient transfection cultures 3 h after replating. In addition, these cultures were compared to mock-transfected cells (data not shown) and to cells transfected with CAP-23, a cortical cytoskeleton–associated protein with structural properties similar to those of GAP-43 (Widmer and Caroni, 1990). When examined with phase-contrast optics, cells expressing high levels of GAP-43 appeared to spread less well than cells expressing little or no GAP-43 (Fig. 2 d). Double labeling of spreading cells for GAP-43 and F-actin revealed that the distribution of subplas-
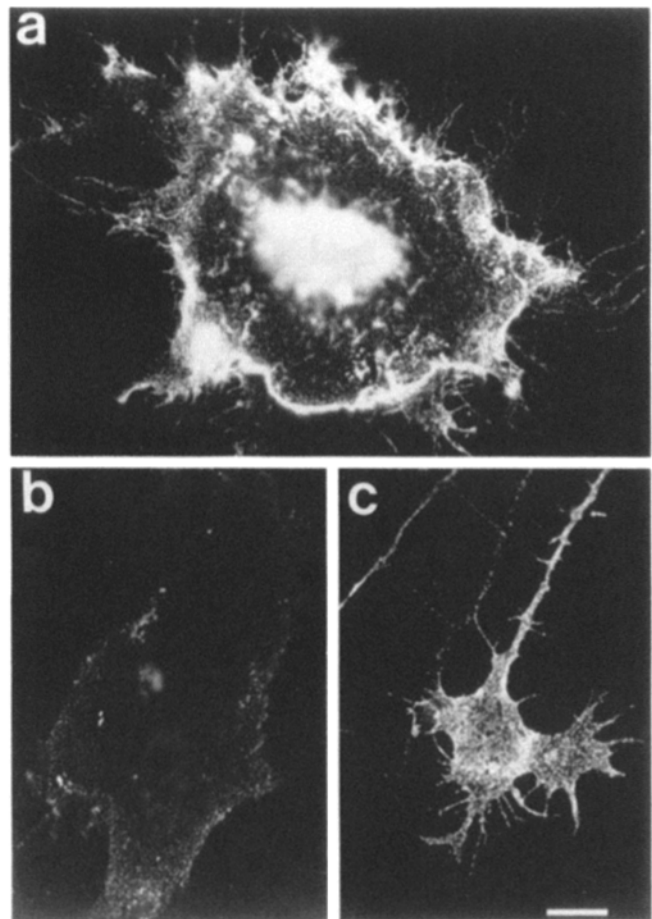

Figure 1. Morphology of L6 cells 2 d after transfection with GAP-43. Cells expressing high levels of GAP-43 displayed numerous filopodia, processlike extensions, and retractions (a), whereas little or no such irregularities were detected in cells expressing low levels of GAP-43 (b). The surface-associated and dotted labeling pattern of GAP-43 in L6 cells resembled that of endogenous GAP-43 in chick dorsal root ganglion neuron growth cones (c). The bright patch at the center of the cell (a) is the cortical cap above the nucleus (out of focus). For these experiments, cells were cultivated on the original substratum for 48 h after transfection before processing for immunocytochemistry. All figures show the distribution of chick GAP-43 as detected with a specific mAb on paraformaldehyde-fixed cells. Bar, 10 μm.

malemmal F-actin was altered in cells expressing high levels of GAP-43. These cells displayed numerous prominent actin-containing filopodia and could easily be distinguished from other cells in the same culture dish by their characteristic intensive spiny and sea urchin–like labeling pattern with F-actin–binding phalloidin (Fig. 2, a and b). These morphological properties and F-actin distribution were not observed in cells expressing high levels of CAP-23 (Fig. 2 c). Furthermore, CAP-23–expressing cells did not display the characteristic filopodia- and actin-rich morphology during earlier phases of spreading (data not shown), indicating that the appearance of GAP-43–expressing cells did not simply reflect a delay in the spreading process. As shown in Table I, the presence of a substantial percentage of cells displaying the radial filopodia- and actin-rich morphology was specifically due to the expression of recombinant wild-type GAP-43, since (a) most cells displaying the characteristic morphology
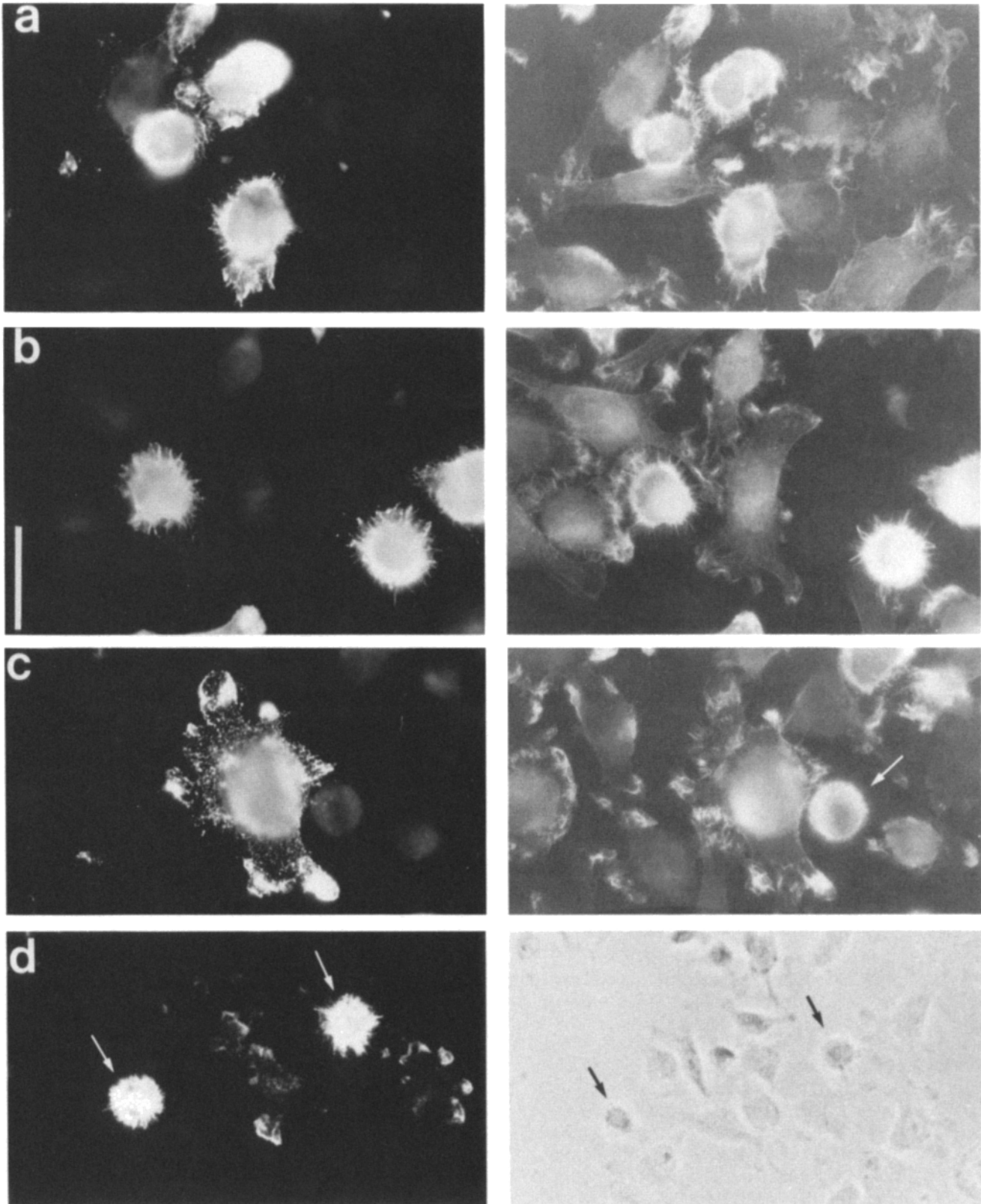
Figure 2. Spreading cells expressing high levels of GAP-43 displayed a filopodia-rich morphology and prominent radial arrays of actin filaments at their periphery. Cultures were transfected with either GAP-43 (*a, b,* or *d*) or with the related cortical cytoskeleton–associated protein CAP-23 (*c*). (*a–c*) Double-labeling experiments showing GAP-43 (*a* and *b*) or CAP-23 (*c*) are on the left and F-actin are on the right. (*d*) Same type of experiment as in *a* and *b*, with GAP-43 on the left and corresponding phase-contrast photograph on the right. Cells were detached from the culture dish 44 h after transfection and fixed with paraformaldehyde 3 h after replating. Note reduced spreading, spiny morphology, and characteristic intensely labeled radial arrays of F-actin–containing filaments in cells expressing high levels of GAP-43 (*arrows*). Also note pseudopodial accumulation of GAP-43 in cells expressing low levels of the transgene (e.g., cell at the right in *d*, not indicated by an arrow). Finally, note that the poorly spread cell indicated by an arrow in *c* did not display the prominent peripheral F-actin labeling pattern of GAP-43–expressing cells. Faint labeling over the nuclei in the left-hand photographs was due to nonspecific background and was also observed when labeling was performed in the absence of mAb. Bar: (*a–c*) 40 μm; (*d*) 60 μm.

Table I. The Presence of Intensely Labeled Radial Arrays of F-actin at the Periphery of Spreading L6 Cells Correlates with the Expression of GAP-43

	Actin++	Transgene++	Transgene++
	% of total	% of actin++	% of total
Wild-type GAP-43	15.0	80.8	12.1
GAP-43(Asp$_{42}$)	5.1	5.8	9.7
CAP-23	5.9	12.1	7.8
Mock transfected	4.8	–	–

Relative contents of cells with prominent radial actin filaments (actin++) in spreading L6 cells transfected with either wild-type GAP-43, a phosphorylation-site mutant of GAP-43, or the cortical cytoskeleton-associated protein CAP-23. Experimental conditions were as described in Fig. 2. Fields were scored first in the RITC channel (F-actin) for actin++ cells (75% of their circumference displayed intensely labeled radial arrays of actin filaments) and total numbers of cells. Corresponding strongly expressing cells (transgene++) were then counted in the Lucifer yellow channel. For comparison, the data shown in Fig. 2 yield the following values: Fig. 2 a: four positive (actin++) cells (the upper two cells are closely spaced), four GAP-43-positive cells (a fifth cell displays low levels and pseudopodial accumulation of GAP-43 immunoreactivity); Fig. 2 b: three positive cells, three GAP-43-positive cells; Fig. 2 c: one positive cell (upper right margin) not corresponding to the CAP-23-positive cell. A total of ~4,000 cells from two separate experiments were scored for each average value given in the figure.

also expressed high levels of GAP-43; and (b) they were found much less frequently in cultures that had been mock transfected, or transfected with either a phosphorylation-site mutant of GAP-43 or with the related cortical cytoskeleton-associated protein CAP-23. Therefore, our data indicate that wild-type GAP-43 affects the morphology and F-actin distribution of spreading L6 cells.

GAP-43 Interacts with Cortical Actin-containing Filaments

At the light microscopic level two patterns of distribution for GAP-43 could be detected: homogeneous dotty distribution under the cell surface and accumulation along cortical actin-containing structures. The latter pattern was particularly prominent in pseudopods (Fig. 3 a; see also Fig. 2, a and d) and at the cortical actin cap above the cell nucleus (Fig. 3 b), but could also be observed along actin-containing protrusions, including filopodia. At close examination, GAP-43 signal along cortical actin structures was also dotted (Fig. 3 b). We did not observe GAP-43 signal along stress fibers; coincidence with actin at pseudopods was restricted to a subset of actin filaments. These observations indicate that GAP-43 that interacted with actin filaments remained associated with the cell surface. In addition, detection of filament-associated GAP-43 was dependent on the presence of intact microfilaments, since disruption of actin filaments with cytochalasin B led to the appearance of the characteristic regularly spaced dotted pattern above the cell nucleus (Fig. 3 c). These experiments suggest that the association of cortical GAP-43 with actin filaments is dynamic: actin-containing structures may retain cortical GAP-43 and high levels of GAP-43 may promote or stabilize cortical actin filaments. On the other hand, the molecular components that interact with GAP-43 in the cell cortex remain to be identified.

Association of GAP-43 with the Cell Cortex is Disrupted by Mutation of Cys$_3$Cys$_4$ to Ala$_3$Ala$_4$

There was no detectable intracellular vesicle-associated or cytosolic GAP-43 in L6 cells, indicating that it efficiently as-
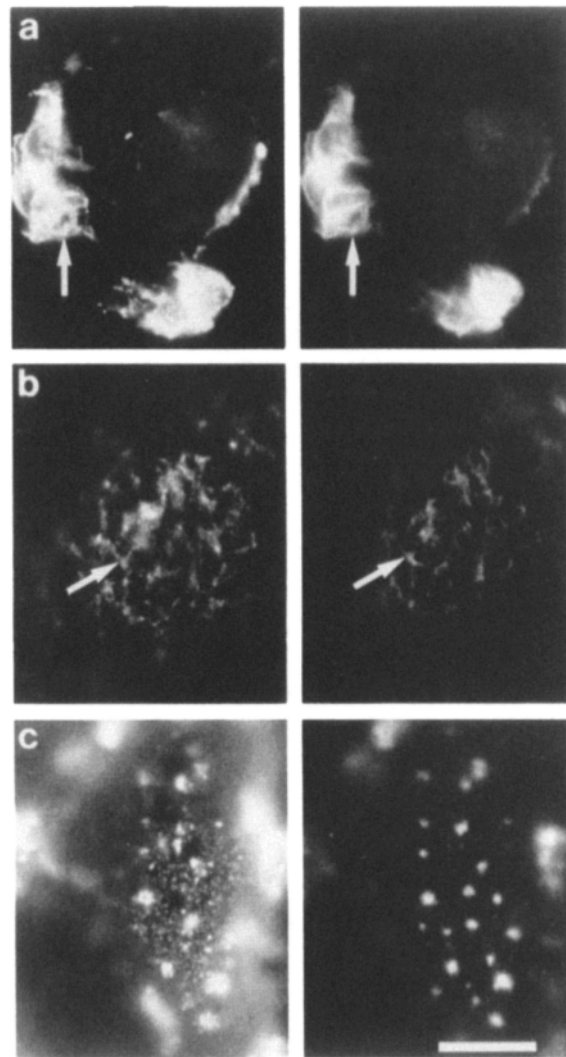

Figure 3. Association of GAP-43 with cortical actin-containing structures. Double-labeling experiments showing GAP-43 are on the left and F-actin are on the right. Cells were fixed with paraformaldehyde 3 h after replating (a) or 2 d after transfection (b and c; experimental conditions are as described in Fig. 1). (a) In spreading L6 cells, GAP-43 accumulated at pseudopods, where it frequently colocalized with actin filaments (arrows). This cell, like those shown in Fig. 2 d, expressed comparatively low levels of GAP-43 and was essentially free of filopodia. (b and c) GAP-43 and F-actin colocalized extensively at the cortical cap above cell nuclei (arrows, b), where the uniformly dotted pattern of GAP-43 signal could not be detected; upon incubation of the cells for 15 min in the presence of 5 μM cytochalasin B (c), F-actin structures collapsed and the dotted GAP-43 pattern could now be detected above the cell nucleus. Bar, 10 μm.

sociated with the cortical cytoskeleton. In spreading cells, transport of GAP-43 to the cell surface may have been predominantly directed to pseudopodial extensions, as these consistently displayed the strongest GAP-43 signals (Fig. 2, a and d, and Fig. 3 a). This may reflect enhanced transport to and incorporation in growing regions of a cell (Bergmann et al., 1983), analogous to neurons, where GAP-43 is rapidly transported to the axonal growth cone in a presumably membrane-bound form (Skene and Willard, 1981; Skene, 1989). In agreement with previous reports indicating that the NH$_2$-terminal region of GAP-43 is responsible for its as-
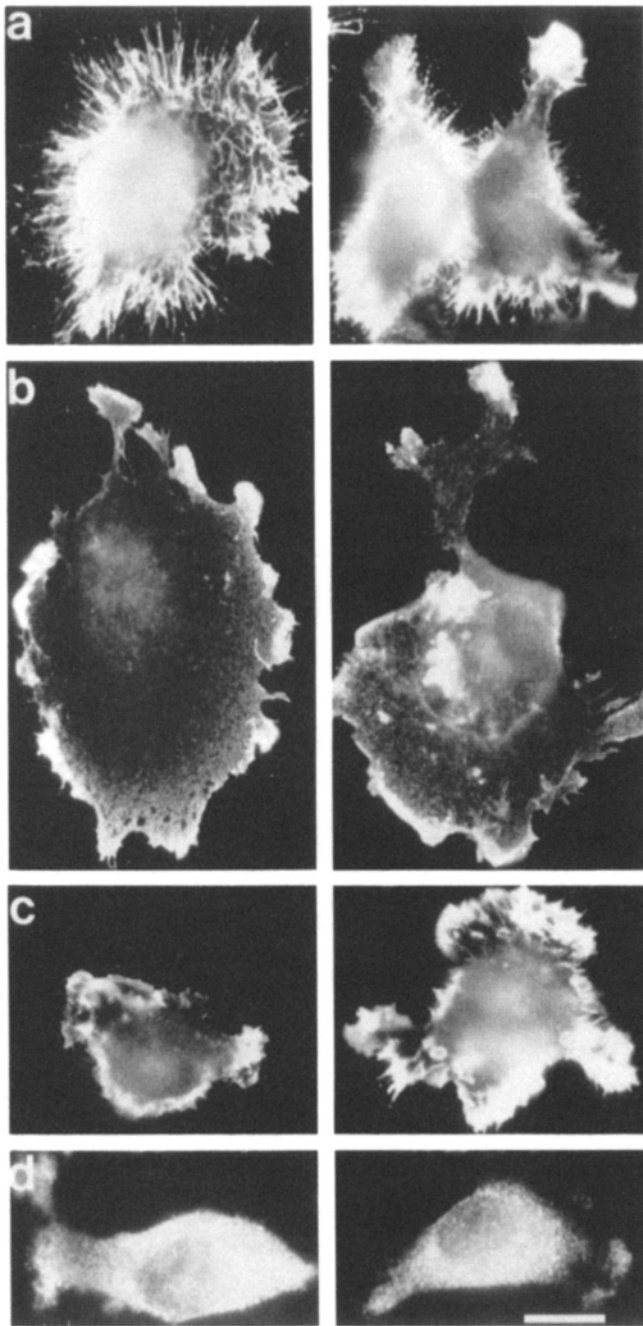
Figure 4. Phenotypes of GAP-43 phosphorylation- and membrane-association mutants. Two examples are shown for each phenotype; these are representative of L6 cells expressing high levels of each mutant. All cells shown were stained for GAP-43. (*a*) Wild-type GAP-43 produced numerous long filopodia and process-like extensions. (*b*) GAP-43(Asp_{42})–expressing L6 cells spread extensively and displayed few filopodia. (*c*) GAP-43(Ala_{42})–expressing L6 cells were small with short filopodia. (*d*) GAP-43(Ala_3Ala_4) yielded a dotted cytosolic labeling pattern and produced no morphological alterations in expressing cells. For these experiments, cells were detached from the culture dish 44 h after transfection and fixed with methanol 4 h after replating. Bar, 10 μm.
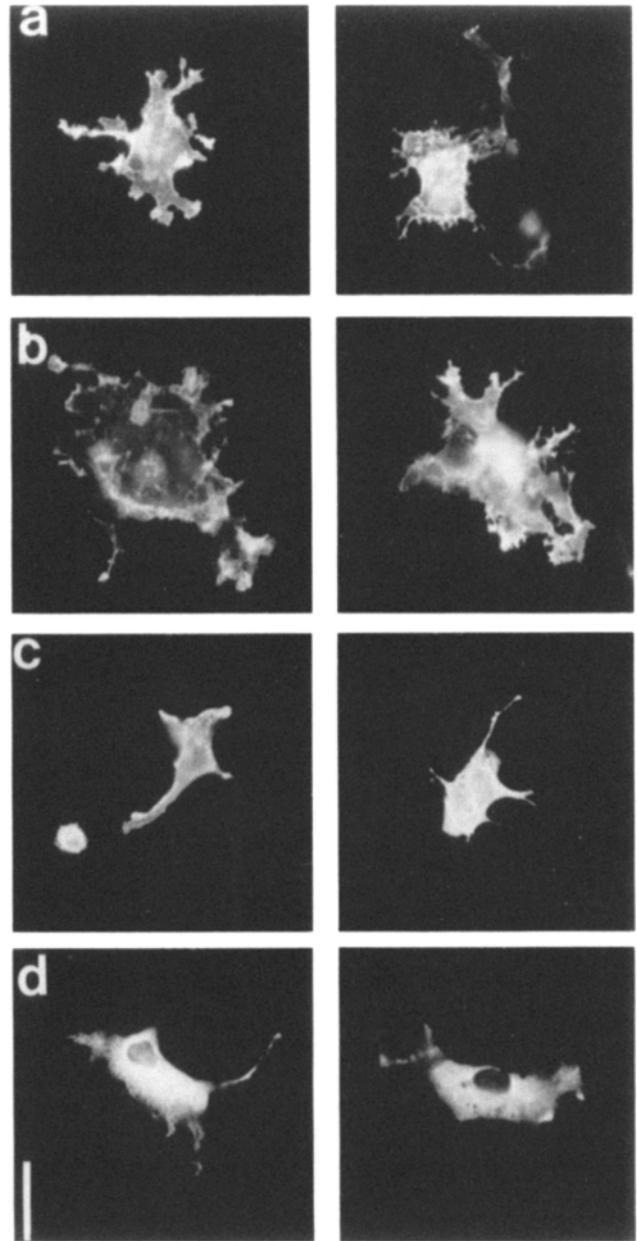

Figure 5. Morphology of spreading COS-7 cells expressing GAP-43 phosphorylation- and membrane-association mutants. Two representative examples are shown for cells expressing high levels of each mutant. All cells were stained for GAP-43. (*a*) Wild-type GAP-43 (irregular outlines, complex morphology); (*b*) GAP-43(Asp_{42}) (large cells, outlines less irregular than in cells expressing wild-type GAP-43); (*c*) GAP-43(Ala_{42}) (small cells, smooth outlines, simple morphology); (*d*) GAP-43(Ala_3Ala_4) (cytosolic labeling pattern, appearance similar to that of nontransfected cells). For these experiments, cells were detached from the culture dish 44 h after transfection and fixed with methanol 6 h after replating. Bar, 45 μm.

sociation with membranes, GAP-43(Ala_3Ala_4), a palmitoylation-negative mutant, yielded a spotted cytosolic labeling pattern (Figs. 4 *d* and 5 *d*). No accumulation of GAP-43(Ala_3Ala_4) in pseudopods was observed. Significantly,

this mutant did not produce the alterations in cell morphology that accompany the expression of the wild-type protein (Fig. 4 and Table II). Therefore, this finding supports the interpretation that the effects of GAP-43 on cell morphology may be based on local interactions with the cell cortex.

Table II. Opposite Effects of GAP-43 Phosphorylation-Site Mutants on Spreading Extent of L6 and COS-7 Cells

	COS-7		L6
	High levels of antigen	Low levels of antigen	High levels of antigen
	spreading areas; $\mu m^2 \times 10^{-2}$		
Wild-type GAP-43	13.4 ± 1.8	9.7 ± 1.5	3.4 ± 0.7
GAP-43(Ala₄₂)	6.9 ± 1.4	10.1 ± 1.7	1.9 ± 0.5
GAP-43(Asp₄₂)	19.6 ± 2.2	13.6 ± 2.0	5.2 ± 1.3
GAP-43(Ala₃Ala₄)	12.4 ± 1.4	–	3.1 ± 0.6
Nonexpressing	10.0 ± 1.6		3.8 ± 1.0
Mock transfected	11.2 ± 1.5		3.9 ± 0.9

44 h after transfection, cells were detached from the culture dish and fixed 4 (L6 cells) or 6 h (COS-7 cells) after replating. After processing for antigen (GAP-43) and F-actin visualization, areas of contact with the substratum were estimated for cells expressing the GAP-43 mutants. Approximately 300 cells from two separate experiments were scored for each value given in the table. Sizes of nonexpressing (GAP-43[Ala₄₂] experiment) and mock-transfected cells were estimated by scoring cells in the RITC channel (F-actin). Average surface values with corresponding standard deviations are given.

The Morphology of GAP-43–transfected Cells Is Modulated by Mutations of the Ser₄₂ Phosphorylation Site

Point mutations at the PKC phosphorylation site of GAP-43 (Ser₄₂ in chick, corresponding to Ser₄₁ in mammalian GAP-43) had major consequences on its effects on cell morphology. L6 cells expressing high levels of GAP-43(Ala₄₂), a mutant that cannot be phosphorylated by PKC, were smaller than average, spread slowly, were poorly polarized, and possessed numerous short filopodia (Figs. 4 c and 5 c; Table II). In contrast, cells expressing high levels of GAP-43(Asp₄₂), a mutant expected to mimic phosphorylated GAP-43, were larger than average, formed prominent process-like irregular extensions, displayed comparatively few filopodia, and appeared to spread more readily than cells transfected with wild-type GAP-43 (Figs. 4 b and 5 b; Table II).

A comparison of spreading L6 cells (Fig. 4) and COS-7 cells (Fig. 5) expressing the various GAP-43 constructs indicate that effects on local surface morphology were similar in both cells. Thus, extensive surface irregularities were observed in COS-7 cells expressing wild-type GAP-43 but not in COS-7 cells expressing GAP-43(Ala₄₂). In addition, cells expressing GAP-43(Asp₄₂) displayed numerous lamellar structures; GAP-43(Ala₃Ala₄) yielded a cytosolic labeling pattern in both L6 and COS-7 cells. While only qualitative comparisons of local surface morphologies between the two cell lines appeared to be possible, very similar effects of the GAP-43 phosphorylation-site mutants were revealed when spreading areas were compared. As shown in Table II, in both L6 and COS-7 cells, GAP-43(Ala₄₂) appeared to prevent cell spreading, whereas GAP-43(Asp₄₂) appeared to promote it. These effects were not detected in cells expressing low levels of the GAP-43 phosphorylation-site mutants (Table II). Similar differences in size were observed during the entire spreading process, although size differences between cells expressing wild-type GAP-43 and GAP-43(Asp₄₂) became less dramatic in well-spread cultures (data not shown).

Evidence indicating that the Ser₄₂ phosphorylation mutants mimicked the behavior of phosphorylated and dephosphorylated GAP-43 is presented in the phorbol ester experi-

ment shown in Fig. 6. Activation of cellular PKC with PMA had the expected consequences on the appearance of cells transfected with the different phosphorylation mutants. In the presence of PMA, GAP-43(Ser₄₂) produced a GAP-43(Asp₄₂)-type of phenotype, whereas the appearance of cells transfected with either GAP-43(Asp₄₂) or GAP-43(Ala₄₂) was little affected. These experiments demonstrate that two different single-amino-acid substitutions of the PKC phosphorylation site of GAP-43 produced distinct effects on its morphological activity, indicating that this property of GAP-43 is modulated by phosphorylation.

Discussion

The results of this study demonstrate that the expression of GAP-43 in nonneuronal cells affects cell surface behavior. Thus, in spreading L6 cells, the presence of high levels of GAP-43 was associated with a filopodia-rich morphology that persisted for several hours and appeared to interfere with spreading and cell polarization. The filopodia-rich morphology and the associated prominent radial arrays of F-actin-containing filaments correlated with the expression of GAP-43 (Fig. 2 and Table II). In addition, they were much less frequent in mock-transfected cells or cells transfected with CAP-23, a cortical cytoskeleton–associated protein with structural and biochemical properties similar to those of GAP-43 (Widmer and Caroni, 1990; Table I).

The effects of GAP-43 on cell surface behavior were apparently not restricted to the process of cell spreading. Thus, well-spread L6 cells expressing high levels of GAP-43 displayed strikingly irregular outlines and numerous long filopodia (Fig. 1 a). COS-7 cells expressing GAP-43 displayed irregular outlines during spreading and in well-spread cultures but produced fewer filopodia than L6 cells. These morphological features were not detected in cells transfected with either CAP-23 or GAP-43(Ala₄₂) and were found much less frequently in cells expressing high levels of GAP-43(Asp₄₂). Similarly, the phosphorylation-site mutants of GAP-43 affected aspects of cell morphology beyond the spreading process: when L6 or COS-7 cells expressing high levels of transgene were compared in 2-d cultures, GAP-43(Asp₄₂) cells were only slightly larger than control or GAP-43 cells, but GAP-43(Ala₄₂) cells had average sizes 50–70% smaller than control cells (data not shown). On the other hand, the prominent peripheral F-actin labeling pattern associated with the expression of GAP-43 in L6 cells was only detected during the first hours of spreading on the substratum. Therefore, in agreement with earlier reports (Zuber et al., 1989), our results indicate that cells are more sensitive to the presence of GAP-43 during spreading. Since spreading of cells and growth cone activity may partly involve similar cellular mechanisms, our observation may be consistent with a proposed role for GAP-43 in neurite elongation (Shea et al., 1991; Yankner et al., 1990). However, recent studies on a PC12 mutant that does not express GAP-43 indicate that this protein is not required for process extension (Baetge and Hammang, 1991). GAP-43 may be an accessory component of the growth cone to mediate responsiveness to second-messenger systems (Skene, 1989). On the other hand, neuronal growth cones also contain the kinase C substrate CAP-23 (Widmer and Caroni, 1990), a GAP-43–like protein displaying a similar cortical localization. Therefore, another possibility is that
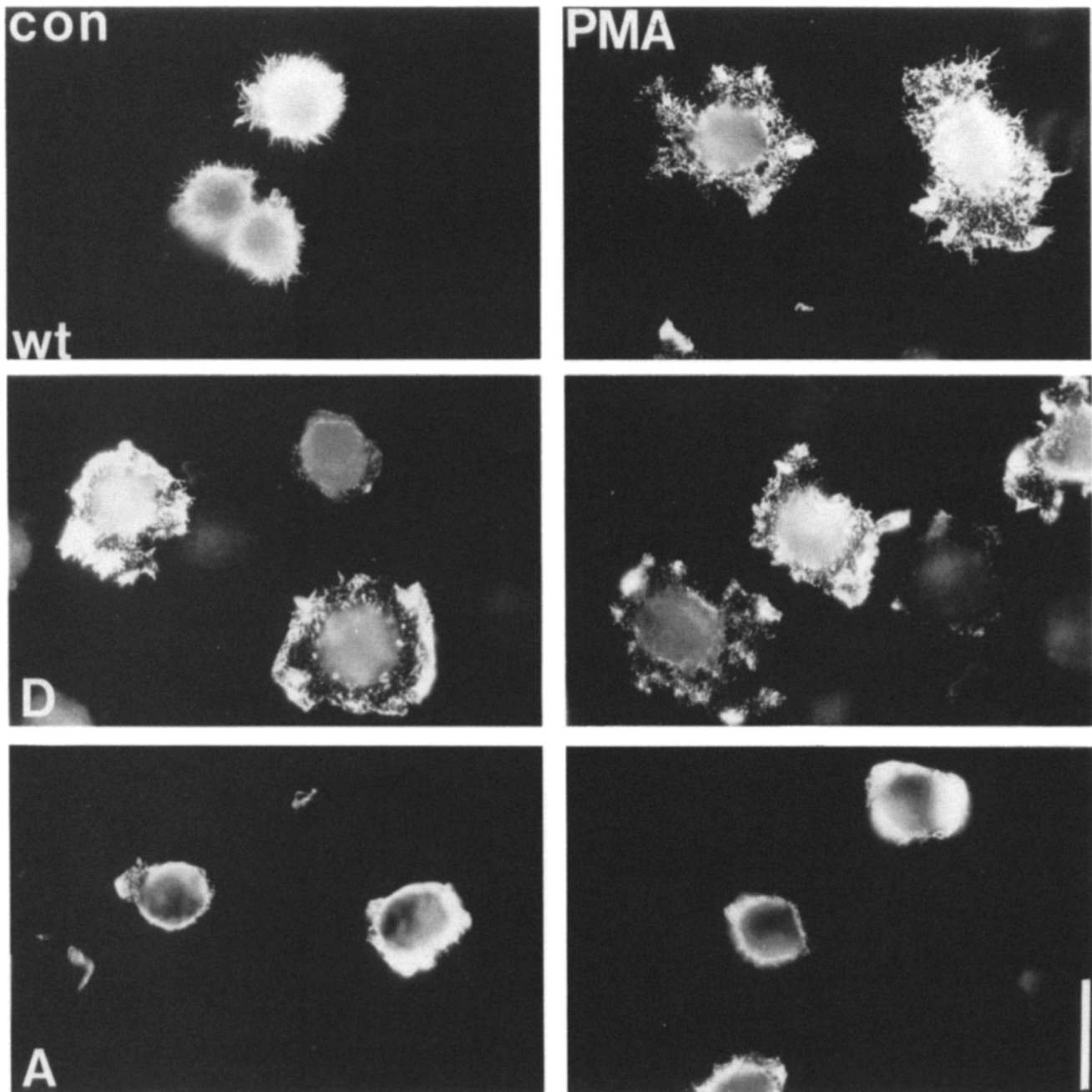
Figure 6. Cells expressing wild-type GAP-43, but not cells expressing GAP-43(Ala$_{42}$), assume a GAP-43(Asp$_{42}$)-type morphology in the presence of the PKC activator PMA. Cells were detached from the culture dish 44 h after transfection, replated, fixed with paraformaldehyde after 1 h, and labeled for chick GAP-43. During the last 30 min before fixation, duplicate dishes were either exposed to solvent (*con*; *left*) or to PMA (*right*; final concentration, 10 nm). *wt*, wild-type GAP-43; *A*, GAP-43(Ala$_{42}$); *D*, GAP-43(Asp$_{42}$). Note that cells expressing wild-type GAP-43 became GAP-43(Asp$_{42}$)-like in the presence of PMA, whereas the phorbol ester had little effect on the appearance of cells expressing either GAP-43(Asp$_{42}$) (mutant-mimicking phosphorylated GAP-43) or GAP-43(Ala$_{42}$) (mutant-preventing PKC-mediated phosphorylation). Bar, 28 μm.

GAP-43 is involved in neurite growth, but that some of its functions may also be performed by other proteins, possibly including CAP-23.

GAP-43 appeared to induce or stabilize radial peripheral actin-containing filaments in spreading L6 cells (Fig. 2 and Table I). Furthermore, the experiments with cytochalasin B (Fig. 3) indicate that GAP-43 may in fact interact with cortical actin-containing filaments. It is therefore tempting to speculate that GAP-43 may directly affect cell cortex be-

havior through its interactions with cell cortex components, including cortical actin-containing filaments.

The phosphorylation-site mutants of GAP-43 displayed distinct effects on local surface morphology and had opposite effects on cell spreading, suggesting that they may represent two functional versions of the molecule. Furthermore, the experiments with PMA support the assumption that GAP-43(Asp$_{42}$) may mimic the behavior of phosphorylated GAP-43. This assumption is further supported by the results of

similar site-directed mutagenesis experiments, where substitution of a serine or threonine with an aspartate or glutamate reproduced properties of the corresponding phosphorylated protein (Thorness and Koshland, 1987; Ducommun et al., 1991; Tavarè et al., 1991). However, we cannot exclude the possibility that GAP-43(Asp$_{42}$) may differ in some important ways from GAP-43 phosphorylated at Ser$_{42}$. While further experiments will be required to clarify this issue, our experiments clearly demonstrate that single-amino-acid substitutions at the PKC phosphorylation site of GAP-43 have major consequences on its effects on cell spreading and morphology. PKC-mediated phosphorylation of GAP-43 is therefore likely to modulate its activity; our findings suggest that the phosphorylation state of GAP-43 at the tip of the axon may affect its tendency to grow.

In addition to their opposite effects on cell spreading, the phosphorylation-site mutants displayed different surface-associated labeling patterns: GAP-43(Asp$_{42}$) labeling tended to be more intense and uniform throughout the cell cortex, whereas GAP-43(Ala$_{42}$) accumulated in cortical actin-rich structures like pseudopodia (data not shown). A marked reduction in the association with cortical actin filaments and the accentuation of the dotted cortical pattern were also observed when cells expressing wild-type GAP-43 were treated with phorbol ester for 5 min, indicating that GAP-43(Asp$_{42}$) reproduced, by this criterion, the behavior of the phosphorylated GAP-43 (data not shown). Conceivably, phosphorylation may promote the association of GAP-43 with the cell cortex. Pseudopodial accumulation of GAP-43(Ala$_{42}$) may be a consequence of preferential incorporation at sites of local membrane growth, followed by poor retention at the cell cortex. Alternatively, GAP-43 may interact with more than one component of the cell cortex and its phosphorylation state may affect the relative strength of these interactions. Cell morphology in our experiments may result from the combined effects of variable intrinsic and environmental factors and the local concentration and phosphorylation status of GAP-43. Clearly, however, it will be essential to identify the mechanisms through which GAP-43 affects cell surface behavior and determine whether this protein is involved directly or indirectly in processes affecting morphogenesis.

Since phosphorylation may modulate the activity of GAP-43, regulation of the corresponding phosphorylating and dephosphorylating enzymes becomes important to its function. Phosphorylation of GAP-43 is specifically dependent on PKC activation (Benowitz and Routtenberg, 1987; Skene, 1989; Coggins and Zwiers, 1991), whereas the calcium- and calmodulin-dependent protein phosphatase calcineurin appears to be a major GAP-43 dephosphorylating enzyme (Liu and Storm, 1989). GAP-43 may therefore respond to both major signaling systems of the growth cone, i.e., regulated and highly localized changes in internal calcium concentration and activation of PKC.

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