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Regulation of GAP-43 at serine 41 acts as a switch to modulate both intrinsic and extrinsic behaviors of growing neurons, via altered membrane distribution

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Abstract

GAP-43 is the major neuronal substrate of protein kinase C (PKC). Its phosphorylation status dictates the severity of pathfinding errors by GAP-43 (+/-) growth cones in vivo, as well as its modulation of actin dynamics in vitro. These experiments show that stably overexpressing cDNAs mutant at its single PKC phosphorylation site at serine41 in retinoic-acid treated SH-Sy5Y neuroblastoma cells regulates intrinsic and extrinsic behaviors of growing neurons. Intrinsically, only Wt and pseudophosphorylated GAP-43Ser41Asp precipitated with F-actin and potentiated F-actin – regulated filopodia formation. GAP-43Ser41Asp inhibited neurite outgrowth whereas only unphosphorylatable GAP-43Ser41Ala precipitated neurotubulin, potentiated neurotubulin accumulation in neurites and increased outgrowth. When PI3-kinase was inhibited GAP-43Ser41Asp-mediated filopodia formation was inhibited whereas GAP-43Ser41Ala-mediated neurite extension was potentiated. Extrinsically, only Wt and GAP-43Ser41Asp potentiated both homotypic adhesion and neurite outgrowth on NCAM-expressing monolayers and promoted NCAM stability. With respect to the underlying mechanism, more F-actin and NCAM colocalized with Wt and GAP-43Ser41Asp in detergent resistant membranes (DRMs) isolated from live cells and GAP-43Ser41Asp-mediated functions were insensitive to cholesterol depletion. In contrast, GAP-43Ser41Ala-mediated functions were sensitive to cholesterol depletion. Neither GAP-43Ser41Asp nor GAP-43Ser41Ala was able to protect against growth cone collapse mediated by PIP2 inhibitors. The results show that modification of GAP-43 at its PKC phosphorylation site directs its distribution to different membrane microdomains that have distinct roles in the regulation of intrinsic and extrinsic behaviors in growing neurons.

Introduction

The ability of growing neurons to respond to directional pathfinding cues is crucially dependent on the balance between regulation of microtubule dynamics in the neurite and actin dynamics within growth cones (Bouquet and Nothias 2007; Pak et al. 2008). In the presence of attractive signals, polymerization of F-actin induces filopodial extension at the leading edge, directing

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growth cone movement toward the guidance cue (e.g. Medalia et al. 2007). Conversely, inhibitory signals induce retrograde flow of F-actin leading to filopodial retraction and growth cone repulsion. The directional response occurs because of reciprocal interactions between the actin and microtubule cytoskeletons and signaling platforms that are established when guidance cue receptors and transduction machinery accumulate at the plasma membrane. Such signaling platforms can be generated by coalescence of highly ordered areas of the membrane enriched in cholesterol, sphingomyelin and phospholipids, termed lipid rafts (Michel and Bakovic 2007). Many growth cone guidance receptors and associated transduction components have structural motifs that target them to lipid rafts (Guirland et al. 2004; Herincs et al. 2005) but how this raft localization translates into directional regulation of growth cone microtubule and actin dynamics is still not well understood.

The growth associated protein GAP-43 is highly enriched in growth cones and targeted to lipid rafts (Meiri et al. 1986; Widmer and Caroni, 1993). It has been described as an intrinsic determinant of growth cone behavior (Wiederkehr et al., 1997) because it modulate levels of cortical F-actin (Caroni 2001). It regulates F-actin in two ways: by binding to F-actin and modulating F-actin dynamics directly (Hens et al. 1993; He et al. 1997; Aarts et al. 1999); and by sequestering the lipid modulator of actin dynamics, PIP(4,5)P2 (PIP2) to inner leaflet rafts via its basic effector (ED) domain (Golub and Caroni 2005; Tong et al. 2008). GAP-43 also modulates microtubule dynamics in the mitotic spindle during neurogenesis (Mishra et al, 2008), but how this may translate into growth cone function has not been explored. GAP-43 is responsive to extrinsic cell-adhesion molecule and neurotrophin-mediated signaling and is required for NCAM mediated neurite outgrowth in vitro (Meiri and Burdick 1991; Meiri et al. 1998; Niethammer et al. 2002; Korshunova et al. 2007; Gupta et al., 2008), but how this sensitivity is coupled to its ability to regulate F-actin in growth cones is not clear.

GAP-43 is the major neuronal substrate of protein kinase C (PKC), and PKC phosphorylation on a single site, serine41, significantly affects GAP-43 interactions with F-actin. In vitro PKC phosphorylated GAP-43 binds directly to F-actin with high affinity (165nM) and prevents filament depolymerization, whereas unphosphorylated GAP-43 also binds F-actin, but with slightly lower affinity (about 2μM) and inhibits filament polymerization (He et al. 1997). These in vitro interactions have direct correlates in living growth cones: areas of growth cones with highly phosphorylated GAP-43 are more adhesive and filopodia rich (Dent and Meiri 1992, 1998), whereas areas with unphosphorylated GAP-43 are dynamic and lamella rich (Meiri et al. 1996). Moreover, productive cell-cell contact stimulates GAP-43 phosphorylation and filopodia formation, whereas non-productive contact is accompanied by dephosphorylation and growth cone collapse (Dent and Meiri 1992, 1998). PKC phosphorylation of GAP-43 also plays an important role in regulating growth cone responses to extracellular axon pathfinding cues *in vivo*. GAP-43 (+/-) cortical growth cones display pathfinding errors whose severity directly correlates with the extent to which residual GAP-43 is phosphorylated by PKC (Shen et al. 2002; McIlvain et al. 2003; McIlvain and McCasland 2006). GAP-43 phosphorylation by PKC can be stimulated by NCAM via activation of the FGF receptor (Meiri et al. 1998; Tejero-Diez et al. 2000), and phosphorylated GAP-43 colocalizes with NCAM in detergent resistant membranes from growth cones thought to be lipid raft equivalents (He and Meiri 2002), and has been shown to regulate neurite outgrowth mediated by NCAM in a raft-dependant fashion (Korshunova et al. 2007), but the role of lipid rafts in coupling GAP-43 phosphorylation in response to NCAM to actin regulation is not clear.

Here we have investigated how modulating the phosphorylation status of GAP-43 in lipid-raft mediated signaling platforms affects actin and microtubule-mediated regulation of intrinsic neuronal functions, namely filopodial extension and neurite outgrowth, and NCAM-mediated regulation of extrinsic functions, namely cell-cell adhesion and neurite outgrowth on a cell-based substrate. We used lines of SH-SY5Y neuroblastoma cells stably expressing GAP-43 a)

behaving as though it is a) constitutively phosphorylated by PKC i.e. ser41 is mutated to asp41 (GAP-43Ser41Asp, Aigner et al. 1995) or b) unphosphorylatable i.e. ser41 is mutated to ala41 (GAP-43Ser41Ala, Meiri et al. 1996). Lipid-mediated signaling platforms were isolated either by extracting live cells in situ to produce membrane patches (Arni et al. 1998) or by extracting cell lysates with detergent and analyzing protein composition biochemically (He and Meiri 2002). Both methods are considered to produce fractions that reflect association of membrane constituents with lipid rafts (Brown 2002, 2007). The results show that expression of constitutively phosphorylated GAP-43 is sufficient to regulate actin-based intrinsic and NCAM-based extrinsic functions, whereas unphosphorylatable GAP-43 is sufficient to regulate microtubule-based intrinsic functions, and that these functions involve association with distinct lipid-mediated signaling platforms.

Methods

Materials

All tissue culture supplies and LipofectAMINE came from GIBCO-BRL (Gaithersburg, MD). The anti- β III tubulin monoclonal or polyclonal antibodies (TuJ-1) was from BABCO (Richmond, CA). The anti caveolin polyclonal antibody was from Transduction Laboratories (San Diego, CA). The anti NCAM monoclonal antibody (HNK-1) and the anti-actin antibodies were from Sigma (St Louis, MO) and the anti-NCAM polyclonal antibody was a generous gift of Dr J. Covault. GAP-43 antibodies were produced in the Meiri lab. Secondary antibodies were from Jackson ImmunoResearch Labs (West Grove, PA). Rhodamine phalloidin was from Molecular Probes (Eugene, OR). Vectashield mountant was from Vector Labs (Burlingame, CA). All other reagents were of the highest quality and were from Calbiochem (San Diego, CA) or Sigma (St Louis, MO).

Plasmids

Rat wt and mutant (Ser41Asp and Ser41Ala) GAP-43 cDNAs have been described previously (Aigner et al. 1995; Meiri et al. 1996), and were subcloned into the mammalian expression vector pCDNA1neo prior to transfection.

Cell Culture and Transfection

SH-SY5Y cells were grown in MEM containing 10% newborn calf serum, 50 U/ml penicillin/streptomycin, and 2 mM glutamine (Wojcikiewicz et al. 1994). For stable transfection, cells in 3.5-cm-diameter dishes (about 80% confluent) were transfected using lipofectAMINE. Stably transfected cells were selected with 500 μ g/ml and maintained with 250 mg/ml Geneticin (G418). Stably transfected lines were subcloned by limiting dilution. Seven independent cell lines expressing either Wt GAP-43 (2 lines) GAP-43Ser41Asp (2 lines) or GAP-43Ser41Ala (2 lines). In addition one line transfected with the plasmid vector pcDNA/neo alone was used as a control.

Pharmacological analyses

Transfected cells were plated on 50 μ g/ml poly-L-lysine coated Labtek slides (Nunc) and treated with 10 μ M retinoic acid for 3 days to initiate neurite extension (Klinz et al. 1987) before treating for 30 minutes with the following stimulators PMA (PKC, 10 μ M) 4 α PMA (PKC control, 10 μ M) or inhibitors Go6976 (PKC, 10 μ M); LY294002 (PI3-kinase, 10 μ M); wortmannin (PIP2, 10 μ M), and/or for 24 hours with the following cholesterol inhibitors: mevalonate (cholesterol, 10 μ M); fumonisins (cholesterol, 10 μ M).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.01% digitonin in 10% goat serum/4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min, and then washed with 0.5% BSA in PBS. For double labeling, cultures were incubated first in primary and secondary antibodies and then in rhodamine phalloidin before coverslipping with Vector mounting medium. For immunocytochemical analysis of detergent insoluble membranes, cells were either rinsed in PBS before fixation with 3% paraformaldehyde or were extracted prior to fixation with 0.1% Triton X-100 in PBS at 4°C as previously described (Arni et al. 1998). Immunocytochemistry used the anti-GAP-43 mab 7B10, the anti-NCAM mab HNK-1, the polyclonal bIII tubulin or anti-caveolin antibodies, followed by Texas-Red or FITC-conjugated anti-mouse IgG or IgM or FITC-conjugated anti-rabbit IgG (Jackson). F-actin was detected with rhodamine phalloidin as before. Cells were visualized with the BioRad MRC-1024 confocal microscope.

Colocalization Analysis Using Confocal Microscopy

Images were acquired sequentially on a Biorad MRC-1024 Confocal Laser microscope using a Kalman filter (N = 4) with settings for gain, iris size, black level, and zoom kept the same across all images. To perform the analysis a single horizontal optical section was first taken through each cell. Then, about 45 vertical sections, each step of which was 0.35 µm, were taken throughout each growth cone at specified points that represent neurites, lamellae and filopodia. Bright yellow pseudocolor indicates that labeling is superimposed. To evaluate colocalization on a pixel-by-pixel basis, LaserSharp version 3.0 software was used to calculate colocalization coefficients that represent the proportion of colocalizing objects in each pixel of a dual-color image. The colocalization coefficients were calculated according to the following equation: $Cred = (\sum Ri, coloc) / (\sum Ri)$. $Ri, coloc$ is the sum of intensities (background subtracted) of all red pixels that also have a green component. Ri is the sum of intensities (background subtracted) of all the red pixels in the image (Manders et al. 1993, Smallcombe and McMillan, 2000)

Calculation of Filopodial Number and Neurite Length

Images of cells labeled with TuJ-1, 7B10 and/or rhodamine phalloidin followed by fluorescent secondary antibodies were photographed following confocal microscopy or fluorescent microscopy. For filopodial analysis, only filopodia longer than 5µm that did not contact other cells were included. Neurite lengths were measured using IPLab software (Scanalytics, Fairfax, VA). For neurite analysis only the longest neurite per cell was included.

Detergent extraction

Extraction of cells to produce detergent resistant membrane microdomains had been described previously. Briefly, cells were extracted with Triton X-114 (TX-114, (He and Meiri 2002) in lysis buffer for 30 min at 4°C at a detergent:protein ratio of 5:1. Lysis buffer contained 150 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1% TX-114, 1 mM PMSF, 1µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml chymostatin, and 0.2 mM sodium orthovanadate. Lysates were passed three times through a 25-gauge needle and then were cleared by 15 min centrifugation at 15,000g. Cleared lysates were subjected to temperature induced phase separation for 5 min at 37°C, followed by centrifugation at 13,000g at room temperature for 3 min. Detergent phases were then precipitated with acetone at -20°C before boiling in 2% SDS buffer (50 mM Tris pH 6.8, 2% SDS). Aqueous phases were precipitated with 10% trichloroacetic acid (TCA); pellets were washed with acetone and boiled in 2% SDS buffer. SDS-PAGE sample buffer (50 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue) was added and samples boiled prior to electrophoresis.

Cross-linking and Immunoprecipitation

In some cases transfected cells were cross-linked by treatment with 0.5mM dithiobissuccinimidyl propionate (DSP) prior to immunoprecipitation (IP) and extraction of DRMs (Safiejko-Mroccka and Bell 1996). Either detergent or aqueous phases from the TX-114 extraction were diluted 1:10 with RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.4, 1mM EDTA, 0.2% Triton X-114, 1mM PMSF, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml chymostatin, and 0.2 mM sodium orthovanadate), precleared with 50 µl of 50% Protein A sepharose, and then centrifuged for 2 min at 270g (Iwabuchi et al. 1998). Supernatants were incubated with the anti-pan-GAP-43 mab 7B10, (Meiri et al. 1991) for 4 hr at 4°C on a rotator. Samples were then incubated with 50 µl of 50% Protein A Sepharose for 2 hr at 4°C. Beads were washed three times with RIPA buffer and once with RIPA buffer (without TX-114), and boiled in SDS-PAGE buffer With 40µM DTT to solubilize immunoprecipitated protein (He and Meiri 2002).

Pelleting of the actin cytoskeleton

For isolation of the actin cytoskeleton, cells were lysed with 0.1% Triton X-100 in PHEM buffer (600 mM Pipes, 250 mM Hepes, 100 mM EGTA, 20 mM MgCl₂, pH 6.9) and F-actin was isolated by centrifugation at 100 000g for 30 minutes at 4°C in the presence of 20µM phalloidin using a Beckman Optima TL ultracentrifuge (Palo Alto, CA) using polycarbonate tubes and a TLA 100 rotor. Triton X-100- insoluble and -soluble fractions were resolved by SDS-PAGE, Western blotted and probed with anti-GAP-43 antibody (Falet et al, 2002).

Western Blots

Transfected cells were lysed in buffer (50 mM Tris-HCl pH 7.4, 2% SDS, 2 mM PMSF), extracted with detergent, or cross-linked and used in immunoprecipitations before solubilization in sample buffer. Proteins were separated on 10% SDS-PAGE gels and then transferred onto PVDF membrane. Immunoreactivity with primary antibodies was detected with HRP-conjugated secondary antibodies and visualized with chemiluminescence.

Other Procedures

Protein concentrations were determined by the Bradford method or by BCA when SDS was present (Pierce, Rockford IL). Bovine serum albumin was used as a standard. For statistical analysis of normally distributed data, ANOVA was first used for analysis of variance, followed by Student's T test to examine statistical significance. Where normal distribution could not be established the Mann-Whitney non-parametric test was used.

Results

Generation of SH-SY5Y lines stably expressing wt and mutant GAP-43 cDNAs

Figure 1 shows clonal lines of human neuroblastoma SH-SY5Y cells stably expressing full-length cDNAs for wt and mutant GAP-43. W8 and W15 express wildtype GAP-43; Asp1 and Asp21 express GAP-43Ser41Asp; Ala32 and Ala44 express GAP-43Ser41Ala; Neo1 expresses pcDNA/NEO vector alone, as control. Endogenous GAP-43 was undetectable in Neo1 cells after 24 hr in culture, however expression was upregulated following treatment with retinoic acid (RA) for 3 days, which induces extension of neurite-like processes Levels of endogenous GAP-43 became maximal after 72 hr of RA treatment (Kim et al. 2000). In contrast each of the transfected cell lines constitutively expressed rat GAP-43 at levels between 3 - 8 fold of maximal endogenous GAP-43, and this was unaffected by RA treatment. Constitutive overexpression of GAP-43 cDNAs had significant effects on cell morphology (see S1). After 3 days of retinoic acid treatment SH-SY5Y cells expressing empty vector elaborated extensive neurites with well-developed growth cones. Neurons expressing GAP-43Ser41Ala were also

able to elaborate significant neurites, however their growth cones were not highly developed. In contrast, neurons expressing either wt or GAP-43Ser41Asp developed growth cones with extensive filopodia, however their neurites were significantly shorter.

Intrinsic behavior I: Phosphorylated GAP-43 potentiates filopodial formation in growth cones in a PI3-kinase dependent fashion, and increases the amount of F-actin associated with detergent resistant membrane patches prepared from live cells

GAP-43 binds directly to F-actin in vitro (He et al. 1997). Figure 2 shows that in SH-SY5Y cells, phospho-GAP-43 (GAP-43Ser41Asp) and unphosphorylatable GAP-43 (GAP-43Ser41Ala) all colocalize with F-actin at the membrane of neurites, growth cone central regions and filopodia. In vitro also, regulation of the PKC phosphorylation site at serine 41 directly affects how GAP-43 interacts with F-actin and modulates F-actin dynamics, such that GAP-43 that is phosphorylated on ser41 stabilizes actin filaments whereas GAP-43 that is not phosphorylated on ser41 acts as a leaky capping protein, inhibiting actin polymerization (He et al. 1997). Figure 3 shows that neurons overexpressing GAP-43Ser41Asp elaborated significantly more filopodia $>5\ \mu\text{m}$ in length per growth cone than those expressing only endogenous or wild type GAP-43. In contrast, filopodia extension was not potentiated in growth cones overexpressing unphosphorylatable GAP-43Ser41Ala. Figure 3 also shows that the filopodia elaborated in the presence of GAP-43Ser41Asp were sensitive to treatment with the PI3-kinase inhibitor LY294002, whereas all filopodia were resistant to treatment with the PKC inhibitor Go 6976.

Treating live cells with TX-100 extracts soluble proteins and a subset of membrane proteins, while leaving detergent resistant membranes (DRMs) intact (see S2, Arni et al. 1998). In untreated (undifferentiated) SH-SY5Y cells, the proportion of cell membrane occupied by GAP-43Ser41Ala DRMs increased 2.8 fold compared with wild type. In contrast the area occupied by GAP-43Ser41Asp DRMs did not increase. Figure 4 shows F-actin colocalization with GAP-43 in DRMs, either calculated by the Pearson coefficient from en face and vertical scans of collapsed confocal Z series sections of after double labeling with GAP-43 antibodies and phalloidin (Fig 4 a-d, see Methods), evaluated by immunoprecipitation after crosslinking DRMs with DSP (Fig 4, c & d, inset) or determined by pelleting with a subcellular fraction containing the actin cytoskeleton (Fig 4e). F-actin colocalized with GAP-43Ser41Asp in 29% of DRMs and coprecipitated with GAP-43 antibody, whereas it colocalized with GAP-43Ser41Ala in only 2.5% of DRMs and did not coprecipitate with GAP-43 antibody. Likewise, only Wt and GAP-43Ser41Asp copelleted with F-actin, whereas GAP-43Ser41Ala did not. Correcting for the total area occupied by DRMs in each cell type indicates that almost 30 fold more F-actin colocalizes with DRMs containing GAP-43Ser41Asp than unphosphorylatable GAP-43Ser41Ala. After 3 days of RA treatment, the levels of F-actin colocalized with all forms GAP-43 in DRMs doubled, even though neither levels of transfected GAP-43 in the DRMs nor the proportion of F-actin associated with GAP-43Ser41Asp compared with GAP-43ser41ala changed.

Intrinsic behavior II: In all cell lines filopodial sensitivity to PIP2 is cholesterol dependent

Fumonisin and mevalonate inhibit cholesterol synthesis and disrupt DRMs (Hering et al., 2003). Figure 5 shows that following fumonisin/mevastatin treatment filopodia formation in GAP-43Ser41Asp-expressing cells shifted from the growth cone to the neurite. Moreover, although the percentage of Neo, wild type or GAP-43Ser41Asp growth cones having filopodia $>5\ \mu\text{m}$ slightly (but not significantly) increased after fumonisin/mevastatin treatment, it was significantly reduced in GAP-43Ser41Ala cells (from 19.75% to 0.04%, ANOVA followed by $p<0.01$ T-test; >100 growth cones counted). In contrast treating cells with wortmannin to inhibit PIP2 had no effect on either GAP-43Ser41Asp-mediated formation of long filopodia or basal levels of short filopodia elaborated by the other transfected lines. However Figure 5 also shows

that when cells in which cholesterol had been depleted were further treated with wortmannin, filopodia formation along GAP-43Ser41Asp neurites were inhibited in and PIP2 also induced collapse of GAP-43Ser41Ala growth cones.

Intrinsic behavior III: Neurites containing phosphorylated GAP-43 are shorter and contain less neurotubulin than those containing unphosphorylated GAP-43. Inhibiting PI3-kinase potentiates the association of neurotubulin with GAP-43Ser41Ala neurites

GAP-43 is upregulated prior to terminal neuronal differentiation and accumulates in the cell body before neurite outgrowth is initiated (e.g. Mishra et al, 2008). Figure 6 shows that significantly more neurotubulin was localized in the neurites of GAP-43Ser41Ala-expressing cells compared with the other cell lines. Moreover when these cells were treated with LY294002 to inhibit PI3-kinase the amount of neurotubulin in the cell bodies and neurites was increased. In contrast wortmannin treatment to inhibit PIP2 had no effect on neurotubulin association with neurites (not shown). Figure 6 also shows that neurites were longer in cells expressing endogenous GAP-43 or GAP-43Ser41Ala compared with those overexpressing either wt or GAP-43ser41asp. When GAP-43Ser41Ala-expressing lines were treated with either LY294002 or Go 6976 significantly neurite length increased significantly. Finally Figure 6 shows that when DRMs extracted from each of the cell lines were immunoprecipitated with the GAP-43 mab and then probed with the β III tubulin mab, significant amounts of β III tubulin were found in the DRM fraction containing GAP-43ser41ala.

Extrinsic behavior I: Phosphorylated GAP-43 potentiates cell-cell adhesion in undifferentiated cells and stimulates neurite outgrowth on cell based substrates

Figure 7 shows en face and vertical scans from collapsed confocal Z series sections illustrating that NCAM and GAP-43 codistribute in neurites as well as cell bodies. Figure 7 also shows that in undifferentiated cells, overexpression of either Wt or GAP-43ser41asp was associated with a significant increase in NCAM localization at the plasma membrane. Moreover homotypic adhesion was only consistently increased between cells expressing wt or GAP-43ser41asp, but not in cells expressing either neo or GAP-43ser41ala. Constitutive overexpression of wild type nor GAP-43Ser41Asp was not sufficient to potentiate neurite outgrowth in RA-treated SH-SY5Y neurons only in the context of endogenous NCAM (see Figure 6). In fact, RA-treated GAP-43Ser41Asp cells had significantly more neurites $<50\mu\text{m}$ in length ($36.7 \pm 7.5\%$) compared with wild type and GAP-43Ser41Ala-expressing cells ($12.5 \pm 7\%$ for WT; $15.8 \pm 5\%$ and $14.7 \pm 6\%$ for GAP-43Ser41Ala). However, when exogenous NCAM was presented by a monolayer of NIH 3T3 cells, neurite outgrowth was significantly stimulated only in the presence of wild type and GAP-43Ser41Asp-expressing SH-SY5Y cells, whereas NCAM was not able to stimulate neurite outgrowth of either Neo or GAP-43Ser41Ala-expressing cells.

Phosphorylation of GAP-43 increases the proportion of SH-SY5Y cells expressing NCAM, and the amount of NCAM associated with detergent resistant membrane patches prepared from live cells

GAP-43 colocalizes with NCAM in a specific subset of DRMS, and NCAM can be coprecipitated from these DRMs by GAP-43 antibody (He and Meiri, 2002). Figure 8 shows colocalization analysis on live cells that have been extracted with TX-100 as before, showing significantly more NCAM colocalized with GAP-43Ser41Asp DRMs than either Wt or GAP-43Ser41Ala. Hence, in untreated cells, NCAM colocalized with GAP-43Ser41Asp in 41% of DRMs, whereas it colocalized with GAP-43Ser41Ala in only 22%. Correcting for the total area occupied by DRMs in each cell type indicates that, as for F-actin, almost 6 fold more NCAM colocalized with DRMs containing GAP-43Ser41Asp than GAP-43ser41ala. After 3 days of RA treatment, the overall level of NCAM colocalized with WT GAP-43 in DRMs

doubled, whereas levels associated with GAP-43Ser41Asp or GAP-43Ser41Ala did not change significantly. SH-SY5Y cells express the 140 kD and 180kD isoforms of NCAM that, like GAP-43, have a palmitoylation motif that targets them to inner leaflet lipid rafts. Western blot analysis to detect specific NCAM isoforms in DRM fractions showed that overexpressing any form of GAP-43 increased the ratio of 180kD/140kD NCAM associating with DRMs in RA treated cells, however this was not sufficient to stimulate neurite outgrowth in the absence of an NCAM monolayer.

Cholesterol-dependence of GAP-43 interactions with DRMs

Although GAP-43 is enriched in cholesterol-containing detergent resistant membranes from cortical growth cones, it is not enriched in caveolae (He and Meiri 2002, but see Joliet et al. 1997). Figure 9 shows that only $22.8 \pm 2.7\%$ of wt GAP-43 colocalized with caveolin-containing DRMs. However, this increased significantly to 43.5% in the presence of GAP-43Ser41Ala. In contrast, expression of GAP-43Ser41Asp had no effect on GAP-43 colocalization with caveolin. On the other hand, treating live cells with mevalonate/fumonisin shows that significantly more DRM protein is resistant to cholesterol extraction in the presence of GAP-43Ser41Asp. GAP-43Ser41Asp DRMs have a more complex protein composition than GAP-43Ser41Ala DRMs and that more proteins directly co-immunoprecipitated from GAP-43Ser41Asp DRMs compared with GAP-43Ser41Ala DRMs. Moreover, when live SH-SY5Y cells are depleted of cholesterol prior to DRM extraction, the resultant GAP-43Ser41Asp DRM population remains complex whereas few proteins remain in GAP-43Ser41Ala DRMs (see S3).

Discussion

These experiments show that stably overexpressing cDNAs mutant at its single protein kinase C phosphorylation site at serine41 can modulate extrinsic as well as intrinsic behaviors of growing neurons. Intrinsically, overexpression of pseudophosphorylated (GAP-43Ser41Asp) in human SH-SY5Y human neuroblastomas potentiated F-actin – regulated filopodia formation at growth cones, whereas unphosphorylatable (GAP-43Ser41Ala) did not. In contrast overexpression of GAP-43Ser41Ala stabilized neurotubulin expression in neurites and potentiated neurite outgrowth whereas GAP-43Ser41Asp did not. Extrinsically, both Wt and GAP-43Ser41Asp increased NCAM stabilization at the membrane, potentiated homotypic adhesion in undifferentiated cells, and increased neurite outgrowth when NCAM was presented as a monolayer substrate whereas GAP-43Ser41Ala did not. With respect to the mechanisms underlying these functions, our results show that PI3 kinase plays distinct and complementary roles in the behavior of GAP-43Ser41Asp and GAP-43Ser41Ala: Only cells expressing GAP-43Ser41Asp responded to PI3-kinase inhibition by decreasing the number of filopodia elaborated. Conversely only cells expressing GAP-43Ser41Ala responded to PI3-kinase inhibitors by increasing the numbers of long neurites. With respect to the underlying structural substrate, detergent resistant membranes isolated from the cells in situ showed significantly more F-actin and NCAM colocalized with Wt and GAP-43Ser41Asp. Following density gradient centrifugation, GAP-43Ser41Asp DRMs from cells were complex and were resistant to cholesterol depletion: When cholesterol synthesis was inhibited in GAP-43Ser41Asp-expressing cells, exogenous filopodial formation along neurites was potentiated. In contrast filopodial formation was inhibited in GAP-43Ser41Ala-expressing cells and growth cones collapsed. Conversely more neurotubulin and caveolin colocalized with GAP-43Ser41Ala DRMs and GAP-43Ser41Ala could immunoprecipitate neurotubulin from DRMs. GAP-43Ser41Ala DRMs were simpler in composition and sensitive to cholesterol depletion (see above). Together the results suggest a model in which the posttranslational modification of GAP-43 on Ser41 acts as switch whereby the unphosphorylated form favors microtubule-based neurite extension and the phosphorylated form favors actin-based neurite stalling and

filopodial formation. Both behaviors are dependent on PI3-kinase, which inhibits GAP-43Ser41Ala-dependent neurite outgrowth and potentiates GAP-43Ser41Asp-dependent filopodial formation.

By ascribing specific roles for phosphorylated and unphosphorylated GAP-43 in regulating the intrinsic and extrinsic functions of growing neurons, in particular F-actin and NCAM-mediated effects (Dent and Meiri 1992, 1998; Meiri et al. 1998; Niethammer et al. 2002), and by demonstrating that the function of both forms of GAP-43 is determined by how it associates with membranes, these results extend previous studies that demonstrated how the intrinsic filopodial formation stimulated when GAP-43 is overexpressed in nonneuronal cells (such as COS-7, L6 cells and fibroblasts (Widmer and Caroni 1993; Aarts et al. 1999) or in neurons of adult transgenic mice (Aigner and Caroni 1993, 1995) requires both Ser41 and the basic PIP2 ED domain (Wiederkehr et al. 1997; Laux et al. 2000). They also extend our previous studies that used a phospho-specific antibody in cultured neurons that correlated phosphorylation status with growth cone behavior in response to extracellular cues (Dent and Meiri 1992, 1998; Meiri et al. 1998). The results also provide new information about GAP-43 interactions with neurotubulin in growing neurites that extend our previous studies focused on the role of GAP-43 at the mitotic spindle (Mishra et al., 2008). Moreover by identifying PI3-kinase as a key underlying pathway in GAP-43 function they extend recent studies describing PI3-kinase-mediated stimulation of filopodia (Luikart et al., 2008).

In living cells the cotranslational palmitoylation of cysteine 3 and 4 that targets GAP-43 to inner leaflet lipid rafts (Arni et al. 1998) also directs it to axons (El-Husseini et al. 2000). About 35% of GAP-43 in cultured neurons is acylated (Liang et al. 2002), and similarly, approximately 50% is associated with the membranes that are resistant to extraction with non-ionic detergent (Triton-X 100 or Triton-X 114) that are thought to operationally represent the association of membrane constituents with lipid rafts (Brown, 1992, 2007) especially when the extraction is performed on live cells in situ rather than on cell homogenates, thereby minimizing artifactual association (He and Meiri 2002, Laux et al. 2000). Such detergent extraction produces fractions of distinct lipid composition that may also be associated with the cortical membrane skeleton (Babiychuk and Draeger 2006), consistent with our previous findings that GAP-43 is associated with a heterogeneous population of DRMs, including those associated with F-actin (He and Meiri 2002), as well as the cortical membrane skeleton itself (Meiri and Gordon-Weeks 1990). Under conditions where the detergent/protein ratio is strictly controlled to standardize extraction, overexpressing GAP-43Ser41Ala was sufficient to increase the proportion of GAP-43 containing DRMs as well as the colocalization of neurotubulin and caveolin with GAP-43 in those DRMs, without increasing the extent that F-actin colocalized with GAP-43 or the ability of F-actin to coprecipitate with GAP-43. Previously, the functional consequences of overexpressing GAP-43Ser41Ala (decreased adhesion and filopodial formation with increased cell spreading) have been ascribed to interactions between GAP-43 and the phospholipid F-actin regulator PIP2 via its effector (ED) domain (Wiederkehr et al. 1997; Laux et al. 2000) and GAP-43 has been shown to sequester PIP2 at lipid bilayers (Tong et al. 2008). Our finding that PIP2 inhibitors cause GAP-43Ser41Ala growth cones to collapse when cholesterol is already depleted extends previous findings that GAP-43Ser41Ala is required for PIP2 and cholesterol-mediated extension of actin lamellae (Golub and Caroni 2005). Moreover by showing that depleting GAP-43Ser41Asp cells of cholesterol induces filopodia formation on neurites that is also PIP2 dependent, they suggest structural compartmentalization can occur between the growth cone and the neurite, even when GAP-43 is constitutively overexpressed. Our results also demonstrate that the ability of GAP-43Ser41Ala to increase the proportion of DRMs in SH-SY5Y cells does not require interactions with actin. In contrast, overexpressing GAP-43Ser41Asp potentiated filopodial formation in growth cones at the same time as significantly increasing F-actin colocalization with GAP-43Ser41Asp in DRMs, consistent with our previous data using the phospho-specific

antibody to show that filopodia formation is accompanied by increased GAP-43 phosphorylation by PKC (Dent and Meiri 1992). The insensitivity of these behaviors to cholesterol depletion, raises the question of how GAP-43Ser41Asp DRMs make specific areas of the growth cone more competent to extend filopodia. The possibility that they represent fragments of the cortical membrane skeleton with which GAP-43 is tightly associated seems to be unlikely since the membrane skeleton fractionates at a much higher sucrose density (>25%, Meiri and Gordon-Weeks, 1990) whereas the complex cholesterol-independent fraction is localized at a much lower density (10% sucrose). GAP-43 is regulated in response to extracellular signals whose receptors are lipid raft components including neurotrophins (Meiri and Burdick 1991; Gupta et al., 2008). The ability of PI3-kinase inhibitors to inhibit GAP-43Ser41Asp-mediated filopodia formation extends recent results describing a role for PI3-kinase in BDNF-mediated filopodial extension (Luikart et al., 2008). Given the ability of GAP-43 to bind directly to PIP3 as well as PIP2 (Nguyen and Meiri, unpublished results) it will clearly be of interest to determine whether the cholesterol-insensitive DRM subfraction containing GAP-43Ser41Asp also contains PIP3. In this regard also, GAP-43 association with sphingomyelin-containing DRMs has been shown in cerebellar granule cells in culture at a time when its phosphorylation in response to extracellular signals is increasing (Palestini et al. 2002). Whether the cholesterol-insensitive GAP-43Ser41Asp DRMs are enriched in sphingomyelin also remains to be seen.

Lipid raft components that regulate GAP-43 include the immunoglobulin superfamily of cell adhesion molecules (Niethammer et al. 2002; Korshunova et al. 2007), hence cerebellar granule cell neurons in which GAP-43 was genetically deleted failed to respond to NCAM-mediated neurite outgrowth signals (Meiri et al. 1998). Manipulating ser41 affected NCAM-mediated functions in a fashion that reflected the increased colocalization of NCAM with GAP-43Ser41Asp in DRMs: Thus overexpressing GAP-43Ser41Asp stimulated NCAM expression at membranes and homotypic adhesion between cells, whereas overexpressing GAP-43Ser41Ala inhibited both. In the same vein GAP-43Ser41Asp neurites were significantly shorter on plastic substrates than either Wt or GAP-43Ser41Ala neurites. On cell-based substrates however (3T3 cells expressing NCAM) NCAM potentiated neurite outgrowth most when GAP-43 was phosphorylatable (i.e. in the Wt form) than when either the pseudophosphorylated or unphosphorylatable form was constitutively present, consistent with data that NCAM signaling actively stimulates GAP-43 phosphorylation by PKC, and suggesting that dynamic regulation of phosphorylation is required for outgrowth to proceed effectively in a situation that more closely resembles the embryonic environment. Retinoic acid treatment of SH-SY5Y cells promotes expression of both NCAM 180 and NCAM 140 although the NCAM 140 form predominates (Seidenfaden et al. 2006). Both NCAM 140 and 180 potentiate neurite outgrowth in a manner dependent on GAP-43 and associated with GAP-43 phosphorylation, but NCAM 180 was more effective in both PC12 and hippocampal neurons (Meiri et al. 1998; Korshunova et al. 2007; Korshunova and Mosevitsky, 2008). Hence, another explanation for the inability of GAP-43Ser41Asp to potentiate neurite outgrowth as much as Wt may be that NCAM 180-mediated pathways are not engaged under conditions where the 3T3 cells are expressing only NCAM 140. In this regard GAP-43Ser41Asp does not specifically increase the ratio of NCAM 180:140 in DRMs, and GAP-43Ser41Asp DRM fractions also show no evidence of the spectrin thought to be required to mediate the interaction between GAP-43 and NCAM (Korshunova et al. 2007). The data here are also consistent with previous results suggesting that in growth cones NCAM association with GAP-43 is likely not due to their direct interaction, but to colocalization in DRMs that is potentiated via a feedback effect caused by phospho-GAP-43 mediated stabilization of the DRMs via F-actin (He et al. 1997; Engelman et al. 1998)

Caveolin is involved in cholesterol-dependent signal transduction and endocytosis via a distinct population of DRMs called caveolae (Engelman et al. 1998; Smart et al. 1999) (Fielding and

Fielding 2000). Likewise GAP-43 also participates in both early stages of endocytosis and vesicle recycling (Neve et al. 1998). Our results here are consistent with previous identification of a subcellular fraction of DRMs distinct from fractions enriched in phosphorylated GAP-43 and F-actin, containing both caveolin and GAP-43 (He et al. 1997). This raises the question of whether the colocalization of caveolin with GAP-43Ser41Ala DRMs reflects the increased endocytosis that accompanies formation of dynamic lamellae.

The cytoarchitecture of the GAP-43 (-/-) CNS is profoundly abnormal *in vivo*, and the severity of the (+/-) phenotype is related to levels of the PKC phosphorylated form in the affected axons (Shen et al. 2002; McIlvain et al. 2003; McIlvain and McCasland 2006). Nonetheless, not all GAP-43 (-/-) axons are affected by absence of GAP-43, particularly in the periphery (Strittmatter et al. 1995). This suggests the presence of GAP-43 independent neurite - outgrowth-promoting mechanisms. One form of GAP-43 independent signaling that is also NCAM-mediated is thought to occur via Src family kinases notably fyn, which interacts with NCAM 140 via receptor tyrosine phosphatase alpha (RPTP α) and also stimulates NCAM-mediated signal transduction via DRMs (Povlsen and Ditlevsen 2008). Our previous results in growth cones show that DRM fractions enriched in phosphorylated GAP-43 do not contain active fyn and vice versa, whereas DRMs enriched in fyn contain unphosphorylated GAP-43 (He and Meiri 2002), raising the question of the role of that the fyn substrate Rac1, also a DRM constituent, may play in triggering actin rearrangements in GAP-43Ser41Ala-containing DRMs (Foger et al. 2001; Ozeri et al. 2001), and whether such GAP-43 independent mechanisms act in parallel with GAP-43-dependent functions, or whether they are spatially or temporally regulated *in vivo*. In this regard it may be pertinent to revisit the notion that transition of phosphorylated GAP-43 into sphingomyelin-associated DRMs is associated with maturation of neurons *in vivo* (Palestini et al. 2002), especially given that expression of phosphorylated GAP-43 correlates with inhibition of neurite outgrowth, that GAP-43 phosphorylation is initiated at the growing tip and then spreads retrogradely into the neurite *in vitro* (Dent and Meiri, 1992) and that phosphorylation is initiated in the growth cone during target identification *in vivo* (Meiri et al. 1991).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aarts LH, Verkade P, van Dalen JJ, van Rozen AJ, Gispen WH, Schrama LH, Schotman P. B-50/GAP-43 potentiates cytoskeletal reorganization in raft domains. *Mol Cell Neurosci* 1999;14:85–97. [PubMed: 10532807]
- Aigner L, Caroni P. Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. *J Cell Biol* 1993;123:417–429. [PubMed: 8408223]
- Aigner L, Caroni P. Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. *J Cell Biol* 1995;128:647–660. [PubMed: 7860637]
- Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, Brenner HR, Caroni P. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell* 1995;83:269–278. [PubMed: 7585944]

- Arni S, Keilbaugh SA, Ostermeyer AG, Brown DA. Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J Biol Chem* 1998;273:28478–28485. [PubMed: 9774477]
- Babiychuk EB, Draeger A. Biochemical characterization of detergent-resistant membranes: a systematic approach. *Biochem J* 2006;397:407–416. [PubMed: 16608442]
- Bouquet C, Nothias F. Molecular mechanisms of axonal growth. *Adv Exp Med Biol* 2007;621:1–16. [PubMed: 18269207]
- Brown DA. Isolation and use of rafts. *Curr Protoc Immunol* 2002;Chapter 11(Unit 11 10)
- Brown DA. Analysis of raft affinity of membrane proteins by detergent-insolubility. *Methods Mol Biol* 2007;398:9–20. [PubMed: 18214370]
- Caroni P. New EMBO members' review: actin cytoskeleton regulation through modulation of PI(4,5)P (2) rafts. *Embo J* 2001;20:4332–4336. [PubMed: 11500359]
- Dent EW, Meiri KF. GAP-43 phosphorylation is dynamically regulated in individual growth cones. *J Neurobiol* 1992;23:1037–1053. [PubMed: 1460463]
- Dent EW, Meiri KF. Distribution of phosphorylated GAP-43 (neuromodulin) in growth cones directly reflects growth cone behavior. *J Neurobiol* 1998;35:287–299. [PubMed: 9622012]
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS. PSD-95 involvement in maturation of excitatory synapses. *Science* 2000;290:1364–1368. [PubMed: 11082065]
- Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, Kohtz DS, Lisanti MP. Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. *FEBS Lett* 1998;428:205–211. [PubMed: 9654135]
- Falet H, Hoffmeister KM, Neujahr R, Hartwig JH. Normal Arp2/3 complex activation in platelets lacking WASp. *Blood* 2002;100:2113–2122. [PubMed: 12200375]
- Fielding CJ, Fielding PE. Cholesterol and caveolae: structural and functional relationships. *Biochim Biophys Acta* 2000;1529:210–222. [PubMed: 11111090]
- Foger N, Marhaba R, Zoller M. Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells. *J Cell Sci* 2001;114:1169–1178. [PubMed: 11228160]
- Golub T, Pico C. Spatial control of actin-based motility through plasmalemmal PtdIns(4,5)P₂-rich raft assemblies. *Biochem Soc Symp* 2005:119–127. [PubMed: 15649136]
- Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ. Lipid rafts mediate chemotropic guidance of nerve growth cones. *Neuron* 2004;42:51–62. [PubMed: 15066264]
- Gupta SK, Mishra R, Kusum S, Spedding M, Meiri KF, Gressens P, Mani S. GAP-43 is essential for the neurotrophic effects of BDNF and positive ampa receptor modulator S18986. *Cell Death & Differentiation*. 2008 In press.
- He Q, Meiri KF. Isolation and characterization of detergent-resistant microdomains responsive to NCAM-mediated signaling from growth cones. *Mol Cell Neurosci* 2002;19:18–31. [PubMed: 11817895]
- He Q, Dent EW, Meiri KF. Modulation of actin filament behavior by GAP-43 (neuromodulin) is dependent on the phosphorylation status of serine 41, the protein kinase C site. *J Neurosci* 1997;17:3515–3524. [PubMed: 9133376]
- Hens JJ, Benfenati F, Nielander HB, Valtorta F, Gispen WH, De Graan PN. B-50/GAP-43 binds to actin filaments without affecting actin polymerization and filament organization. *J Neurochem* 1993;61:1530–1533. [PubMed: 8377002]
- Herincs Z, Corset V, Cahuzac N, Furne C, Castellani V, Hueber AO, Mehlen P. DCC association with lipid rafts is required for netrin-1-mediated axon guidance. *J Cell Sci* 2005;118:1687–1692. [PubMed: 15811950]
- Hering H, Lin CC, Sheng M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J Neurosci* 2003;23:3262–71. [PubMed: 12716933]
- Iwabuchi K, Handa K, Hakomori S. Separation of “glycosphingolipid signaling domain” from caveolin-containing membrane fraction in mouse melanoma B16 cells and its role in cell adhesion coupled with signaling. *J Biol Chem* 1998;273:33766–33773. [PubMed: 9837965]

- Joliot A, Trembleau A, Raposo G, Calvet S, Volovitch M, Prochiantz A. Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties. *Development* 1997;124:1865–1875. [PubMed: 9169834]
- Kim SN, Kim SG, Park SD, Cho-Chung YS, Hong SH. Participation of type II protein kinase A in the retinoic acid-induced growth inhibition of SH-SH-SY5Y human neuroblastoma cells. *J Cell Physiol* 2000;182:421–428. [PubMed: 10653609]
- Klinz FJ, Yu VC, Sadee W, Costa T. Differential expression of alpha-subunits of G-proteins in human neuroblastoma-derived cell clones. *FEBS Lett* 1987;224:43–48. [PubMed: 3119368]
- Korshunova I, Novitskaya V, Kiryushko D, Pedersen N, Kolkova K, Kropotova E, Mosevitsky M, Rayko M, Morrow JS, Ginzburg I, Berezin V, Bock E. GAP-43 regulates NCAM-180-mediated neurite outgrowth. *J Neurochem* 2007;100:1599–1612. [PubMed: 17212696]
- Korshunova I, Mosevitsky M. Role of the Growth-associated Protein GAP-43 in NCAM-mediated Neurite Outgrowth. *Neurochem Res*. 2008 Aug 6; 2008. Epub ahead of print.
- Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P. GAP43, MARCKS, and CAP23 modulate PI (4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J Cell Biol* 2000;149:1455–1472. [PubMed: 10871285]
- Liang X, Lu Y, Neubert TA, Resh MD. Mass spectrometric analysis of GAP-43/neuromodulin reveals the presence of a variety of fatty acylated species. *J Biol Chem* 2002;277:33032–33040. [PubMed: 12105219]
- Luikart BW, Zhang W, Wayman GA, Kwon CH, Westbrook GL, Parada LF. Neurotrophin-dependent dendritic filopodial motility: a convergence on PI3K signaling. *J Neurosci* 2008;28:7006–12. [PubMed: 18596174]
- Manders EE, Verbeek FJ, Aten JA. Measurement of co-localisation of objects in dual-colour confocal images. *J Microscopy* 1993;169:375–382.
- McIlvain V, McCasland JS. GAP-43 heterozygous mice show delayed barrel patterning, differentiation of radial glia, and downregulation of GAP-43. *Anat Rec A Discov Mol Cell Evol Biol* 2006;288:143–157. [PubMed: 16435363]
- McIlvain VA, Robertson DR, Maimone MM, McCasland JS. Abnormal thalamocortical pathfinding and terminal arbors lead to enlarged barrels in neonatal GAP-43 heterozygous mice. *J Comp Neurol* 2003;462:252–264. [PubMed: 12794747]
- Medalia O, Beck M, Ecker M, Weber I, Neujahr R, Baumeister W, Gerisch G. Organization of actin networks in intact filopodia. *Curr Biol* 2007;17:79–84. [PubMed: 17208190]
- Meiri KF, Gordon-Weeks PR. GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. *J Neurosci* 1990;10:256–266. [PubMed: 2137164]
- Meiri KF, Burdick D. Nerve growth factor stimulation of GAP-43 phosphorylation in intact isolated growth cones. *J Neurosci* 1991;11:3155–3164. [PubMed: 1834809]
- Meiri KF, Pfenninger KH, Willard MB. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc Natl Acad Sci U S A* 1986;83:3537–3541. [PubMed: 3517863]
- Meiri KF, Bickerstaff LE, Schwob JE. Monoclonal antibodies show that kinase C phosphorylation of GAP-43 during axonogenesis is both spatially and temporally restricted in vivo. *J Cell Biol* 1991;112:991–1005. [PubMed: 1705561]
- Meiri KF, Hammang JP, Dent EW, Baetge EE. Mutagenesis of ser41 to ala inhibits the association of GAP-43 with the membrane skeleton of GAP-43-deficient PC12B cells: effects on cell adhesion and the composition of neurite cytoskeleton and membrane. *J Neurobiol* 1996;29:213–232. [PubMed: 8821178]
- Meiri KF, Saffell JL, Walsh FS, Doherty P. Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *J Neurosci* 1998;18:10429–10437. [PubMed: 9852580]
- Michel V, Bakovic M. Lipid rafts in health and disease. *Biol Cell* 2007;99:129–140. [PubMed: 17064251]

- Neve RL, Coopersmith R, McPhie DL, Santeufemio C, Pratt KG, Murphy CJ, Lynn SD. The neuronal growth-associated protein GAP-43 interacts with rabaptin-5 and participates in endocytosis. *J Neurosci* 1998;18:7757–7767. [PubMed: 9742146]
- Mishra R, Gupta SK, Meiri KF, Fong M, Thostrup P, Juncker D, Mani S. GAP-43 is key for mitotic spindle control and centrosome-based polarization in neurons. *Cell Cycle* 2008;7:348–357. [PubMed: 18235238]
- Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. *J Cell Biol* 2002;157:521–532. [PubMed: 11980923]
- Ozeri V, Rosenshine I, Ben-Ze'Ev A, Bokoch GM, Jou TS, Hanski E. De novo formation of focal complex-like structures in host cells by invading *Streptococci*. *Mol Microbiol* 2001;41:561–573. [PubMed: 11532125]
- Pak CW, Flynn KC, Bamberg JR. Actin-binding proteins take the reins in growth cones. *Nat Rev Neurosci* 2008;9:136–147. [PubMed: 18209731]
- Palestini P, Botto L, Guzzi F, Calvi C, Ravasi D, Masserini M, Pitto M. Developmental changes in the protein composition of sphingolipid- and cholesterol-enriched membrane domains of rat cerebellar granule cells. *J Neurosci Res* 2002;67:729–738. [PubMed: 11891786]
- Povlsen GK, Ditlevsen DK. The Neural Cell Adhesion Molecule NCAM and Lipid Rafts. *Neurochem Res*. 2008
- Safiejko-Mroccka B, Bell PB Jr. Bifunctional protein cross-linking reagents improve labeling of cytoskeletal proteins for qualitative and quantitative fluorescence microscopy. *J Histochem Cytochem* 1996;44:641–656. [PubMed: 8666749]
- Seidenfaden R, Krauter A, Hildebrandt H. The neural cell adhesion molecule NCAM regulates neuritogenesis by multiple mechanisms of interaction. *Neurochem Int* 2006;49:1–11. [PubMed: 16469417]
- Shen Y, Mani S, Donovan SL, Schwob JE, Meiri KF. Growth-associated protein-43 is required for commissural axon guidance in the developing vertebrate nervous system. *J Neurosci* 2002;22:239–247. [PubMed: 11756507]
- Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PE, Okamoto T, Lisanti MP. Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol* 1999;19:7289–7304. [PubMed: 10523618]
- Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell* 1995;80:445–452. [PubMed: 7859286]
- Tanaka S, Tominaga M, Yasuda I, Kishimoto A, Nishizuka Y. Protein kinase C in rat brain synaptosomes. Beta II-subspecies as a major isoform associated with membrane-skeleton elements. 1: *FEBS Lett* 1991;294:267–70.
- Tejero-Diez P, Rodriguez-Sanchez P, Martin-Cofreces NB, Diez-Guerra FJ. bFGF stimulates GAP-43 phosphorylation at ser41 and modifies its intracellular localization in cultured hippocampal neurons. *Mol Cell Neurosci* 2000;16:766–780. [PubMed: 11124896]
- Tong J, Nguyen L, Vidal A, Simon SA, Skene JH, McIntosh TJ. Role of GAP-43 in sequestering phosphatidylinositol 4,5-bisphosphate to Raft bilayers. *Biophys J* 2008;94:125–133. [PubMed: 17827240]
- Widmer F, Caroni P. Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J Cell Biol* 1993;120:503–512. [PubMed: 8421062]
- Wiederkehr A, Staple J, Caroni P. The motility-associated proteins GAP-43, MARCKS, and CAP-23 share unique targeting and surface activity-inducing properties. *Exp Cell Res* 1997;236:103–116. [PubMed: 9344590]
- Wojcikiewicz RJ, Tobin AB, Nahorski SR. Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca²⁺ and by rapid desensitization. *J Neurochem* 1994;63:177–185. [PubMed: 8207426]

Figure 1. Expression of GAP-43 by stably transfected SH-SY5Y cells

Western blot probed with the mab 7B10 that recognizes all isoforms of GAP-43. Neo1: expressing empty vector. Others, subcloned lines. W15 and W8: expressing Wt GAP-43. Asp1 and Asp21: expressing GAP-43Ser41Asp. Ala32 and Ala44: expressing GAP-43Ser41-ala. First 2 lanes: Neo and W15 cells treated with 10 μ M RA for 3 days to induce neurite outgrowth. Upper arrow: endogenous (human) GAP-43. Lower arrow transfected (rat) GAP-43. Other lanes without RA; 10 μ g protein in each lane.

Figure 2. GAP-43 and F-actin colocalization in three dimensions in growth cones

Confocal micrographs of RA-treated, stably transfected lines probed with anti-GAP-43 mab (green) and rhodamine phalloidin (red). Double labeling is in yellow. Top panels show a single horizontal optical section through the whole growth cone. Lower panels in each figure show vertical Z series taken throughout at specific points (i, ii, iii, iv). Each step 0.35 μm, about total 45 vertical sections. Scale bar: 20 μm.

Figure 3. Filopodial formation by growth cones

Percent of growth cones from RA-treated stably transfected lines with >4 filopodia 5μm long. >100 growth cones from 3 independent treatments counted. Percentage increased in Wt and GAP-43Ser41Asp vs. Neo ($p < 0.05$, and $p < 0.01$; ANOVA followed by 1 tailed T-test) and in Wt vs. GAP-43Ser41Asp ($p < 0.01$, ANOVA followed by 1 tailed T-test). Percent decreased in GAP-43Ser41Asp vs. Neo following treatment with 10μM LY294002.

Figure 4. GAP-43 and F-actin colocalization in detergent-resistant membranes

Stably transfected lines were either rinsed with PBS (a) or extracted with 0.1% Triton X-100 at 4°C prior to fixation (b-d) and labeling with the GAP-43 mab 7B10 and rhodamine phalloidin. Bright yellow pixel:pixel colocalization was calculated from sequential confocal images. a) Neo-transfected cells before extraction show little colocalization because endogenous GAP-43 is low before RA treatment b) Wt15-expressing cells with increased extent of GAP-43 colocalization with F-actin in DRMs. c) GAP-43Ser41Ala-expressing cells show little colocalization. Inset; IP with GAP-43 probed with 7B10 (top panel) and actin antibody (bottom panel) showing no actin co-IP with GAP-43Ser41Ala. d) GAP-43Ser41Asp-expressing cells show enhanced colocalization. Inset; IP with GAP-43 probed with 7B10 (top panel) and

actin antibody (bottom panel) showing actin co-IP with GAP-43 Ser41Asp. Scale Bar = 20 μ m. e) Both Wt and GAP-43Ser41Asp copellet with the F-actin cytoskeleton, whereas GAP-43Ser41Ala does not. Western blot probed with the anti-GAP-43 mab 7B10 (arrow). T = total protein, p = pellet. 45 μ g total lysate used for each experiment. f) Quantitation showing significantly more colocalization of F-actin with GAP-43Ser41Asp in DRMs ($p < 0.001$, ANOVA followed by 2 tailed T-test $n > 100$ growth cones from each line measured). g) The experiment was repeated with neurons after 3 days of RA treatment. ($p < 0.001$, ANOVA followed by 2-tailed T-test $n > 100$ neurons from each line measured).

Figure 5. Effects of cholesterol depletion on filopodia formation

a – d) RA-treated stably transfected lines incubated for 24 hours in mevalonate and fumonisins before fixing and staining with rhodamine phalloidin. a) GAP-43Ser41Asp expressing cells show enhanced filopodia formation along neurites (arrows). b) GAP-43Ser41Ala expressing cells show inhibition of filopodia formation in growth cones (arrows). c) GAP-43Ser41Asp-expressing neurons also show increased branching (arrow). d) Wt-expressing cells show little effect on filopodia formation. Scale bar = 2 μ m. e) Quantitation of the decrease in filopodia formation in growth cones. n>100 growth cones from each line measured. (f – i) mevalonate and fumonisins-treated cells further incubated with wortmannin to inhibit PIP2 (f, g) Neo and Wt-expressing neurites show increased branching. (h) Filopodia on GAP-43Ser41Asp-expressing neurites collapsed (arrows) (i) Collapse of GAP-43Ser41Ala expressing growth cones (arrow) with residual filopodia (asterisk). Scale bar = 4 μ m.

Figure 6. βIII tubulin expression and regulation of neurite length

9 (a-f) Micrographs of RA-treated, stably transfected lines probed with anti-βIII tubulin mab (green) and rhodamine phalloidin (red). Left hand side panels show increased βIII tubulin reactivity in growth cones from GAP-43Ser41Ala-expressing cells (arrow c) compared with Wt (arrow a). Right hand side panels show potentiation of βIII tubulin reactivity when cells are treated with LY294002. g) Percent of neurites from RA-treated stably transfected lines >200μm long after treatment with 10μM LY294002 or 100nM Go 6976. Percentage was increased in GAP-43Ser41ala cells ($p < 0.01$; ANOVA followed by 2 tailed T-test). $n > 100$ growth cones from 3 independent treatments counted. h) Western blot of DRMs from transfected lines (top) probed with the βIII tubulin antibody TuJ-1; (bottom) immunoprecipitated with the GAP-43 mab 7B10 before probing with TuJ-1. More βIII tubulin is present in IPs from GAP-43Ser41ala DRMs than in the other cell lines.

Figure 7. GAP-43 and NCAM colocalization in three dimensions – effects on cell adhesion and neurite outgrowth

a – c) Confocal micrographs of RA-treated, stably transfected lines probed with anti-GAP-43 mab (red) and the NCAM polyclonal antibody (green). Double labeling is in yellow. Top panels show a single horizontal optical section through the whole growth cone. Lower panels in each figure show vertical Z series taken throughout at specific points (i, ii). Arrows show that colocalization is limited to the middle sections corresponding to an intracellular distribution. Each step 0.35 μ m, about total 45 vertical sections. Scale bar: 20 μ m. d) Stably transfected lines were fixed and labeled with NCAM antibody and the percent of total cells expressing NCAM at the plasma membrane was quantitated in each line. The percentage was significantly increased in either Wt or GAP-43Ser41Asp expressing cells ($p < 0.01$, ANOVA followed by 1 tailed-T test) whereas it was decreased in GAP-43Ser41Ala expressing cells ($p < 0.05$, ANOVA followed by 1 tailed-T test). $N > 100$ cells from each line counted. e) Stably transfected lines were plated at low density and left overnight before counting the number of groups of >10 cells in each line. Both Wt and GAP-43Ser41Asp expression significantly enhanced cell-cell adhesion ($p < 0.05$, ANOVA followed by 1 tailed-T test, and $p < 0.001$ ANOVA followed by 2 tailed-T test respectively) $n > 50$ fields from each line measured. f) Fold stimulation of neurite length, when cells were grown on a monolayer of NIH 3T3 cells expressing NCAM $140 \pm$ RA. Neurites in each of 8 fields were measured and averaged. RA- treated neurites were

significantly longer in Wt and GAP-43Ser41Asp expressing lines ($p < 0.005$, 2-directional Mann Whitney test).

Figure 8. GAP-43 and NCAM colocalization in detergent-resistant membranes

Stably transfected undifferentiated (a, b) or RA-treated (c-f) lines extracted with 0.1% Triton X-100 at 4°C prior to fixation and labeling with the GAP-43 mab 7B10 and NCAM. Bright yellow pixel:pixel colocalization calculated from sequential confocal images. a) GAP-43Ser41Asp expressing cells showing high levels of colocalization and cell-cell adhesion. b) GAP-43Ser41ala expressing cells. c) Neo transfected cells showing intracellular colocalization d) Wt-expressing cells showing colocalization at the membrane, neurites and growth cones. e) GAP-43Ser41ala expressing cells showing little colocalization. f) GAP-43Ser41Asp expressing cells showing colocalization at the membrane, neurites and growth cones. Scale bar = 50µm. g,h) Quantitation. g) Significantly more colocalization of

NCAM with GAP-43Ser41Asp compared with wt in non-RA treated DRMs ($p < 0.01$, ANOVA followed by 2-tailed T-test $n > 100$ growth cones from each line measured). h) 3 days after RA treatment, no significant difference compared with Wt. i) Western blot showing NCAM distribution in the detergent (D) and aqueous phases (A) after DRM isolation with TX-114 of RA-treated cells, 5 μ g of protein loaded in each lane. Arrow 180kD NCAM isoform. j) Ratio of NCAM 180/140 distribution in DRMs: An increase with all forms of GAP-43, but no significant differences between them.

Figure 9. GAP-43 and caveolin colocalization in DRMs from neurites

(a – d). Colocalization of GAP-43 and caveolin in DRMs from transfected RA-treated SH-SY5Y cells. Colocalization images were acquired with Biorad LaserSharp software to using dual-color images and subtracting background. a) Neo transfected cells. b) Wt15 c) Asp1 Caveolin (green) labeled intracellularly (arrows in 4× inset). d) Ala32. Caveolin is associated with GAP-43 at the cell membrane (arrows in 4× inset). e, f) Pixel by pixel colocalization analysis form single confocal section, as before. e) Asp 1. f) Ala 32. Scale bar = 10 μm. g) Quantitation of colocalization. Significantly more GAP-43Ser41ala colocalized with caveolin in neurites ($p < 0.05$, ANOVA followed by 2 tailed T-test $n > 100$ growth cones from each line measured).

Figure 10. Composition of detergent-resistant membranes

Silver-stained 10% gels of protein from RA-treated GAP-43Ser41Asp and GAP-43Ser41ala cells. a) GAP-43Ser41Asp DRMs are more complex when equivalent amounts of starting material are loaded. b) More complex protein profile immunoprecipitated by GAP-43 mab from GAP-43Ser41Asp DRMs. Arrowhead 43kD actin band. c) DRM protein remaining when cholesterol synthesis is depleted by mevalonate/fumonisin treatment. Arrowhead 43kD actin band. MW 100kD, 75kD, 50 kD, 37 kD, 20 kD.