Posttranslational Membrane Attachment and Dynamic Fatty Acylation of a Neuronal Growth Cone Protein, GAP-43

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Abstract. Growth cones, the motile apparatus at the ends of elongating axons, are sites of extensive and dynamic membrane–cytoskeletal interaction and insertion of new membrane into the growing axon. One of the most abundant proteins in growth cone membranes is a protein designated GAP-43, whose synthesis increases dramatically in most neurons during periods of axon development or regeneration. We have begun to explore the role of GAP-43 in growth cone membrane functions by asking how the protein interacts with those membranes. Membrane-washing experiments indicate that mature GAP-43 is tightly bound to growth cone membranes, and partitioning of Triton X-114–solubilized GAP-43 between detergent-enriched and detergent-depleted phases indicates considerable hydrophobicity. The hydrophobic behavior of the protein is modulated by divalent cations, particularly zinc and calcium.

In vivo labeling of GAP-43 in neonatal rat brain with [^{35}S]methionine shows that GAP-43 is initially synthesized as a soluble protein that becomes attached to membranes posttranslationally. In tissue culture, both rat cerebral cortex cells and neuron-like PC12 cells actively incorporate [^{3}H]palmitic acid into GAP-43. Isolated growth cones detached from their cell bodies also incorporate labeled fatty acid into GAP-43, suggesting active turnover of the fatty acid moieties on the mature protein. Hydrolysis of ester-like bonds with neutral hydroxylamine removes the bound fatty acid and exposes new thiol groups on GAP-43, suggesting that fatty acid is attached to the protein's only two cysteine residues, located in a short hydrophobic domain at the amino terminus. Modulation of the protein's hydrophobic behavior by divalent cations sugests that other domains, containing large numbers of negatively charged residues, might also contribute to GAP-43–membrane interactions. Our observations suggest a dynamic and reversible interaction of GAP-43 with growth cone membranes.

GAP-43 is a major protein component of the motile growth cones that form the advancing tips of growing axons (Meiri et al., 1986; Skene et al., 1986). Although the cellular roles of GAP-43 have not been established, elevated synthesis of the protein is strongly correlated with developmental axon outgrowth (Skene and Willard, 1981*b*; Kalil and Skene, 1986; Jacobson et al., 1986) and with successful axon regeneration (Skene and Willard, 1981*a*, *b*; Benowitz and Lewis, 1983). A subset of synaptic terminals contain high concentrations of GAP-43 in adult brain (Oestreicher and Gispen, 1986; Oestreicher et al., 1986; Benowitz et al., 1988; McGuire et al., 1988), where phosphorylation of GAP-43 (also designated F1, B-50, or p57) has been correlated with long term potentiation of synaptic transmission (Routtenberg, 1986; Lovinger et al., 1986). Phosphorylation of GAP-43 by protein kinase C is correlated with translocation of kinase C from cytosol to synaptic membranes during long term potentiation (Akers and Routtenberg, 1987), and phosphorylated GAP-43 has been reported to modulate phosphoinositide metabolism in synaptic membranes (Oestreicher et al., 1983; van Dongen et al., 1985). Storm and colleagues have proposed that an important role of GAP-43 is to sequester calmodulin along the cytoplasmic surface of appropriate membrane domains (Andreason et al., 1983; Alexander et al., 1987). It is important, therefore, to understand how GAP-43 becomes associated with growth cone and synaptic membranes, and how that membrane attachment may be regulated.

A previous study indicated that GAP-43 remains bound to membranes under high salt conditions and in the presence of divalent cation-chelating agents, suggesting strong binding of the protein to membranes (Skene and Willard, 1981*c*). Analysis of the protein's sequence shows no potential membrane-spanning domains (Basi et al., 1987; Karns et al., 1987; Rosenthal et al., 1987), but there is a small hydrophobic domain at the amino terminus of GAP-43 that might serve as a hydrophobic anchor for other membrane-binding moieties added posttranslationally (Basi et al., 1987). A number of proteins have been found to contain covalently bound lipid, either myristic acid attached cotranslationally as an NH_2-terminal amide, or longer chain fatty acids attached to proteins posttranslationally, predominantly through thioester

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linkages to cysteine residues (Sefton and Buss, 1987; Burn, 1988). Because the putative membrane-binding domain of GAP-43 occurs at the amino terminus of the protein and also contains two cysteine residues (Basi et al., 1987), we have considered the possibility that GAP-43 attachment to membranes involves posttranslational addition of fatty acids.

Materials and Methods

In Vivo Labeling of Rat Brain Proteins

Sprague Dawley rats, 3–11 days of age, were anesthetized on ice. [^{35}S]Methionine (>800 Ci/mmol, Amersham Corp., Arlington Heights, IL) or [^{35}S]cysteine (>600 Ci/mmol, Amersham Corp.) were injected with a Hamilton Co. (Reno, NV) syringe either intraperitoneally or intracranially (directly through the skull at three to four sites). Total radioactivity injected ranged in various experiments from 200 to 1,000 μCi, in a maximum volume of 50 μl. After survival times ranging from 10 min to 18 h, the animals were killed with carbon dioxide and their brains removed.

In Vitro Labeling of Cortical Cultures and Growth Cones

Trypsin-dissociated primary cultures of cerebral cortex from embryonic day 16 rats were prepared according to Booher and Sensenbrenner (1972) except that they were seeded onto polylysine-coated plastic in DME supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 mg/ml). Dissociated cells were maintained 24 h in an atmosphere of 92% O_2/8% CO_2 before labeling. Intact growth cone particles were isolated from 4-d-old rat brains according to the method of Gordon-Weeks and Lockerbie (1984) except that the first Ficoll gradient centrifugation was increased to 18,000 rpm for 20 min and the final flotation gradient omitted (Gordon-Weeks, 1987). Both cortical cell cultures and growth cones were labeled with 200 μCi of either [^{3}H]palmitic acid (50 Ci/mmol, Amersham Corp.) or [^{3}H]myristic acid (40 Ci/mmol, Amersham Corp.) for 1–2 h in 3 ml of unsupplemented DME. Individual samples contained ~1 × 10^6 cortical cells, or intact growth cones from one to two brains. Cells or growth cones were resuspended by trituration, pelleted by centrifugation at 500 *g* for 5 min, and washed with fresh medium. Cells or growth cones were lyzed in a hypoosmotic lysis buffer containing 10 mM Tris, pH 7.5, 5 mM EDTA and the following protease inhibitors: antipain (10^{-6} M), aprotinin (1.4 × 10^{-6} M), bacitracin (10^{-5} M), benzamidine (10^{-3} M), benzethonium choloride (10^{-4} M), leupeptin (10^{-5} M), 1,10-*O*-phenanthroline (1 mg/ml), pepstatin A (10 mg/ml), phenylmethyl sulfonyl fluoride (10^{-3} M), soybean trypsin inhibitor (5 × 10^{-6} M).

In some cases, labeled samples were collected by centrifugation at 10,000 *g* for 5 min and washed five times with 10 ml of chloroform/methanol (2:1) to remove lipids not bound to protein. The remaining pellets were air dried, dissolved in 1% SDS, and heated 5 min at 100°C, and analyzed by electrophoresis.

Preparation of Soluble and Membrane Fractions

Tissues and cultured material were homogenized in osmotic lysis buffer in a Potter-Elvejehm homogenizer. Nuclei and cell debris were removed by centrifugation at 5,000 *g* for 5 min. The low speed supernatant was subjected to centrifugation at 100,000 *g* for 20 min. Unless otherwise indicated in the text "soluble fraction" refers to the high speed supernatant, and "membrane fraction" refers to the pellet of this initial high speed centrifugation.

Detergent Partitioning of Proteins

A stock of Triton X-114 was prepared by precondensing four times from an initial 2% (wt/vol) aqueous solution as described by Bordier (1981). Membrane samples were solubilized on ice in the indicated buffers containing 1% (wt/vol) Triton X-114, and insoluble material removed with centrifugation at 100,000 *g* for 20 min. Samples were incubated 2 min at 30°C and centrifuged at 12,000 *g* for 2 min in a prewarmed rotor. The detergent-depleted ("aqueous") upper phase and the detergent-enriched ("detergent") lower phase were carefully separated. Additional detergent or buffer was added to each fraction so that the final detergent concentration was 1%, and the partitioning repeated. The four resulting samples were adjusted to equal volumes, and equal aliquots removed for electrophoresis.

Immunoprecipitation of GAP-43

Anti-GAP-43 monoclonal antibody (91E12) was preincubated with goat anti-mouse-coupled beads (Hyclone Laboratories, Logan, UT) in IP buffer (10 mM Sodium phosphate, pH 7.5, 0.9% NaCl, 5 mM EDTA, and 0.5% Nonidet P-40) for 2 h at 4°C with constant rotation. The beads were then washed three times in 1 ml IP buffer, recovered by centrifugation, and resuspended in 1 ml IP buffer. Protein samples in 1% SDS were added to a final concentration of <0.1% SDS, incubated 2 h, and washed as above. The resulting immune complexes were solubilized by 3 min at 100°C in 1% SDS.

Electrophoresis

Proteins were analyzed on one-dimensional gels containing 12% acrylamide/0.36% bisacrylamide using the buffer system of Laemmli (1970) or on two-dimensional gels run essentially according to O'Farrell (1975), as previously described (Jacobson et al., 1986). IEF gels contained a pH 3.5–10 ampholines and pH 4–6 ampholines mixed in a 1:1 ratio. Gels were fixed and stained with Coomassie Brilliant Blue R according to Fairbanks et al. (1971) or silver stained as described by Morrisey (1981). Gels were prepared for fluorography using the APEX system (Jen and Thach, 1982), except that samples labeled with ^{3}H-fatty acids were prepared for fluorography as described by Bonner and Laskey (1974) to avoid the high concentration of acetic acid used in the APEX procedure. In more recent experiments, we have used the APEX procedure successfully to detect fatty acid–labeled proteins. Gels were exposed to x-ray film preexposed to a background optical density of 0.1 (Laskey and Mills, 1975). Quantitation of fluorographs and autoradiographs were performed by optical densitometry, using a two-dimensional densitometric scanner (Technology Resources, Nashville, TN).

To analyze the lability of the GAP-43–palmityl bond to neutral hydroxylamine, samples were subjected to one-dimensional PAGE and the gels soaked 4 h at room temperature in at least 10 vol of 1 M Tris, pH 7.0, or 1 M hydroxylamine, pH 7.0. The gels were then washed for 1 h in water and prepared for fluorography.

For the immunoblotting experiments illustrated here, proteins were transferred from SDS–polyacrylamide gels to nitrocellulose as previously described (Jacobson et al., 1986), by soaking gels first for 2 h in 10% trichloroacetic acid, 10% acetic acid, and 20% isopropanol; and then for 2 h in 25 mM Tris base, 192 mM glycine, and 1% SDS. Electrophoretic transfer was then carried out for 16 h at 30 V at 4°C in a transfer buffer containing 20 mM Tris base, 150 mM glycine, 20% methanol, and 0.05% SDS. Similar results have been obtained without presoaking the gels, using the high pH carbonate transfer buffer described by Dunn (1986). Blots were probed with a monoclonal antibody (91E12) which recognizes both the primary translation product of GAP-43 mRNA and mature GAP-43 isolated from growth cones. Bound antibody was detected with biotinylated goat anti-mouse IgG and avidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA), using 4-chloro-1-napthol as the chromophore in the peroxidase colorimetric reaction.

Proteolytic Mapping

For chymotryptic mapping of GAP-43–linked fatty acid, growth cones were isolated from 5-d-old rat pups injected intracranially with [^{35}S]cysteine 24 h earlier, or from unlabeled animals. Growth cones from unlabeled animals were labeled for 2 h *in vitro* with [^{3}H]palmitic acid as described above. Membrane fractions were separated on two-dimensional gels and GAP-43 localized by staining the gels 1 h in Coomassie Blue. Gels were destained 30 min in 10% isopropanol and washed in 1 liter of deionized water. Gel regions containing GAP-43 were excised and subjected to chymotryptic digestion essentially as described by Cleveland et al. (1977). Briefly, the gel pieces were washed 30 min in 1 ml of 125 mM Tris, pH 7.5. The gel pieces were then loaded into the sample wells of a 20% PAGE gel (4% acrylamide in the stacking gel), and overlaid with sample buffer (125 mM Tris, pH 7.5, 1 mM EDTA, 10% glycerol, trace of bromophenol blue) containing either 1 or 4 μg of chymotrypsin. Control samples were treated identically but overlaid with buffer containing no chymotrypsin. Electrophoresis was carried out at 60 V. The gels were stained with Coomassie Blue and prepared for fluorography.

Thin-layer Chromatography

Growth cones were labeled with [^{3}H]palmitic acid and the labeled proteins

Figure 1. Extraction of GAP-43 from growth cone membranes. Growth cones from neonatal (4-d) rat cerebral cortex were lyzed in 10 mM Tris, pH 7.5, 5 mM EDTA and centrifuged 20 min at 100,000 *g*. The membrane pellet was then washed sequentially in a series of buffers containing 10 mM Tris, pH 7.5, 5 mM EDTA, and the indicated additions. Equal aliquots of each supernatant were analyzed for GAP-43 by immunoblotting. (Lane *1*) Initial supernatant; (lane *2*) 1 M NaCl; (lane *3*) a second wash in 1 M NaCl; (lane *4*) 150 mM NaCl/1% Tween-20; (lane *5*) Tris/EDTA alone; (lane *6*) Triton X-114–insoluble material; (lane *7*) Triton X-114–soluble material.

separated by two-dimensional electrophoresis. GAP-43 was localized by staining the gels with Coomassie Blue. Gel pieces containing GAP-43 were excised, and washed first in deionized water and subsequently in methanol. The gel pieces were incubated 4 h in 500 ml of 0.1 M KOH in methanol. The supernatant was removed and extracted with 2 vol of chloroform/water (1:1); the upper (aqueous) phase was reextracted with 1 vol of chloroform. The organic phases were combined and washed three times with chloroform/methanol/water (1:10:10). The final organic phase was dried, redissolved in chloroform/methanol/water (65:25:4), and spotted onto a silica thin-layer plate previously developed with acetone and dried. The sample lane was flanked by lanes containing palmitic acid and palmitic acid–methyl ester standards. Chromatography was in chloroform/methanol/water (65:25:4), and lipid standards were visualized by exposure to iodine vapor for 5 min at 37°C. 1-cm-wide segments of the sample lane were scraped from the plate and counted in a scintillation counter.

Quantitation of Free Thiol Groups

Growth cone membranes were prepared as described above and dissolved in 1% SDS. 100-μl aliquots were removed and dialyzed for 3 h against 100 ml of 1 M hydroxylamine, pH 7.0, or 1 M Tris, pH 7.0, at room temperature. Samples were subsequently dialyzed for 1 h each against 100 mM Tris, pH 8, and then against 0.1 M Tris, pH 8, containing 6 M guanidine hydrochloride. 10 μCi of [^{14}C]iodoacetamide (50 mCi/mmol, Amersham Corp.) in 6 M guanidine HCl/0.1 M Tris, pH 8, was incubated with each sample for 2 h at room temperature in the dark. Samples were then dialyzed against 1% SDS, 0.1 M Tris, pH 8, 5 mM dithiothreitol to remove unreacted iodoacetamide. Equal aliquots of each sample were then analyzed by two-dimensional electrophoresis. Gels were stained with Coomassie Blue, dried, and exposed to x-ray film.

Results

Membrane Attachment and Hydrophobic Properties of Mature GAP-43

To assess the nature of GAP-43 attachment to growth cone membranes, we prepared a subcellular fraction containing intact growth cones from neonatal rat brain (Gordon-Weeks and Lockerbie, 1984; Gordon-Weeks, 1987). The isolated growth cones were lyzed in a low ionic strength buffer and the membranes washed with a series of buffers to remove loosely bound proteins. After each wash, membranes were recovered by centrifugation at 100,000 *g* for 20 min, and the supernatant removed for later analysis of extracted proteins. Consistent with earlier results with regenerating toad nerves (Skene and Willard, 1981*c*) the major form of GAP-43 from neonatal rat brain remains associated with growth cone membranes through serial washes at low ionic strength, high ionic strength (1 M NaCl), and in the presence of a divalent cation chelator (EDTA). GAP-43 also remained bound to membranes washed in the presence of the surfactant Tween-20 (Fig. 1). The majority of GAP-43 was removed from the membrane pellet only upon solubilization of the membranes with nonionic detergent (Triton X series), suggesting that the major form of GAP-43 is tightly bound to membranes. Similar results were obtained when all wash buffers contained either 1 mM calcium or 1 mM zinc instead of EDTA (not shown).

Persistent association of GAP-43 with membranes under a variety of ionic conditions suggested that hydrophobic interactions might play an important role in the protein's attachment to growth cone membranes. We used detergent-phase partitioning to assess the hydrophobic behavior of GAP-43 solubilized from growth cone membranes. Membranes were washed as described above and solubilized in Triton X-114. Detergent-soluble material was warmed briefly to 30°C to aggregate detergent micelles, permitting separation of a detergent-enriched phase and a detergent-depleted ("aqueous") phase by low speed centrifugation (Bordier, 1981; Pryde, 1986). Each fraction was readjusted to the starting concentration of detergent and repartitioned. In the presence of EDTA, membrane-derived GAP-43 behaved as an amphipathic protein, partitioning approximately equally between detergent and aqueous phases at low ionic strength (Fig. 2). The proportion of GAP-43 in the detergent phase increases slightly when the ionic strength is increased (150 mM NaCl). The protein partitions predominantly into the detergent phase in the presence of divalent cations (1 mM Ca^{+2} and Mg^{+2}) at approximately physiological ionic strength (150 mM NaCl; Fig. 2).

An initial survey indicates some differences in the modulation of the hydrophobic behavior of GAP-43 by various divalent cations. At low total ionic strength, partitioning of GAP-43 into the detergent phase was enhanced by 1 mM Zn^{+2} (Fig. 2), but 1 mM Mg^{+2} or Ca^{+2} had little effect (not shown). However, calcium does appear to promote more hydrophobic behavior of GAP-43 at concentrations between 10 and 100

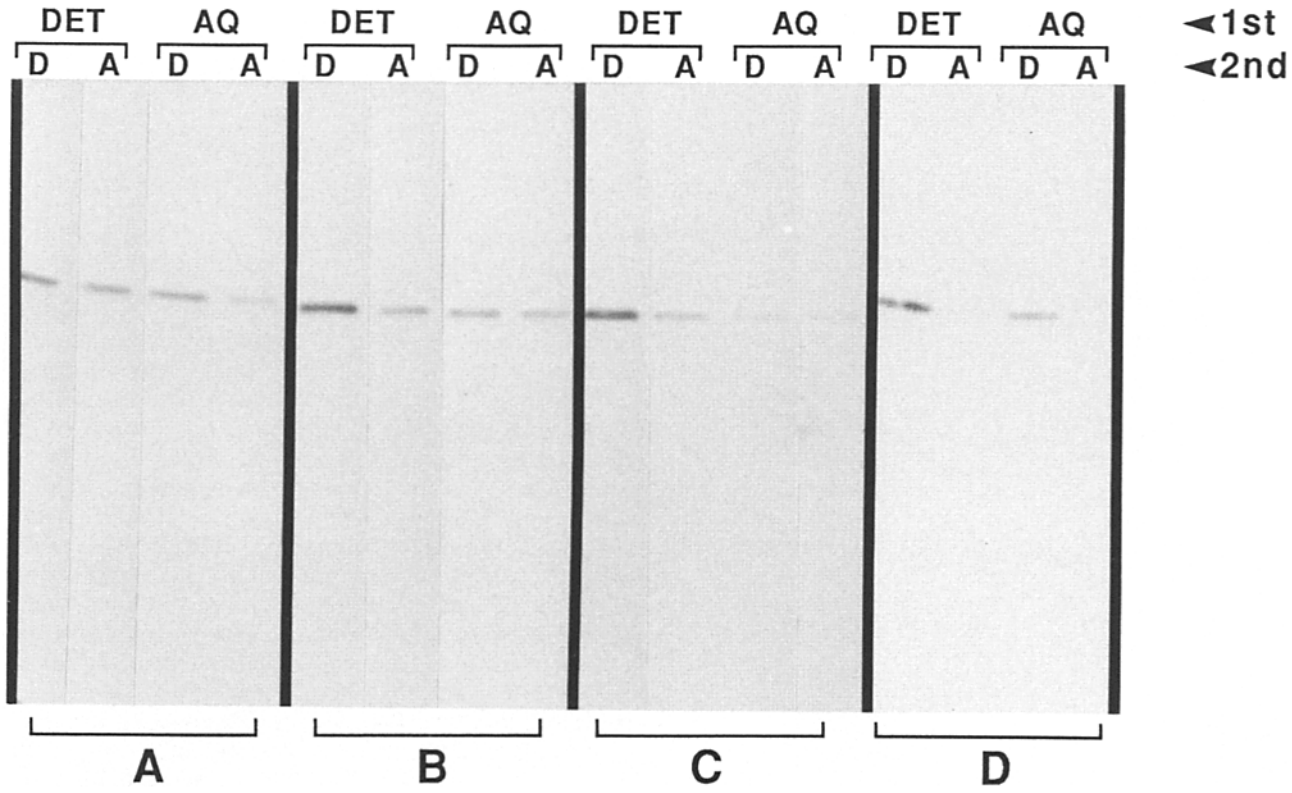

Figure 2. Detergent-phase partitioning of GAP-43. Growth cone membranes were washed as described for Fig. 1 and solubilized in >10 vol of buffer containing 1% (wt/vol) Triton X-114. A detergent-enriched phase (*Det*) and a detergent-depleted "aqueous" phase (*Aq*) were separated; each of these fractions was readjusted to 1% Triton X-114 in the original volume and the partitioning repeated. The resulting detergent (*D*) and aqueous (*A*) fractions were adjusted to the same volume and equal aliquots analyzed for GAP-43 by immunoblotting. Phase separations were carried out in the following buffers: (*A*) 10 mM Tris/5 mM EDTA; (*B*) 10 mM Tris/5 mM EDTA/150 mM NaCl; (*C*) 10 mM Tris/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$; (*D*) 10 mM Tris/1 mM $ZnCl_2$.

μM, while partitioning of GAP-43 was similar at zinc concentrations of 1.0 and 0.1 mM (not shown). We have not conducted a systematic exploration of ion-specific modulation of GAP-43–detergent interactions.

Our observations suggest that the attachment of GAP-43 to growth cone membranes involves hydrophobic interactions between the membrane bilayer and either GAP-43 itself or another hydrophobic moiety to which GAP-43 is strongly bound.

Posttranslational Attachment of GAP-43 to Membranes

Because the predicted amino acid sequence of GAP-43 contains no apparent domains capable of hydrophobic interaction with membranes, we considered the possibility that the protein's ability to interact strongly with membranes might be acquired through posttranslational modification. Metabolic labeling of GAP-43 in intact rat brain indicates that GAP-43 is indeed synthesized as a soluble protein that becomes attached to membranes posttranslationally.

We labeled newly synthesized GAP-43 in neonatal rat cerebral cortex by either intraperitoneal or intracranial injection of [^{35}S]methionine. Intracranial injection provides rapid delivery of label to the brain, while intraperitoneal injection provides more uniform and reproducible labeling for quantitative comparisons between animals. Using either injection route, newly synthesized GAP-43 can be detected within 20 min of injection, at which time a large fraction of the newly synthesized GAP-43 can be recovered in a high speed supernatant ("cytosol") from cerebral cortex (Figs. 3 *A* and 4). The soluble GAP-43 can be precipitated specifically by a monoclonal antibody raised against mature GAP-43 (Fig. 3 *A*). With intracranial injection, labeled GAP-43 could be detected in the soluble fraction 10 min after labeling, at which time no labeled GAP-43 could be detected in the membrane fractions (Fig. 4 *A*). Absolute labeling of GAP-43 in the soluble fraction increases for ~40 min after intraperitoneal injection of [^{35}S]methionine and then declines, concomitant with increasing labeling of GAP-43 in the membrane fraction (Fig. 5). We therefore suggest that GAP-43 is synthesized in neonatal cortex as a soluble protein that becomes associated with membranes posttranslationally.

Both soluble and membrane-bound GAP-43 appear on two-dimensional gels as single spots, comigrating with the primary translation product, at all times sampled over the first 2 h after labeling (Fig. 4). Subsequently, membrane-bound GAP-43 appears as a series of spots more acidic than the primary translation product (Fig. 4 *B*). The exact number and distribution of these spots is somewhat variable, but by 24 h after injection, the distribution of labeled spots coincides with the positions of the silver-stained mature forms of GAP-43 from growth cone membranes (illustrated in Fig. 3 *B*). Mature GAP-43 from growth cone membranes can be

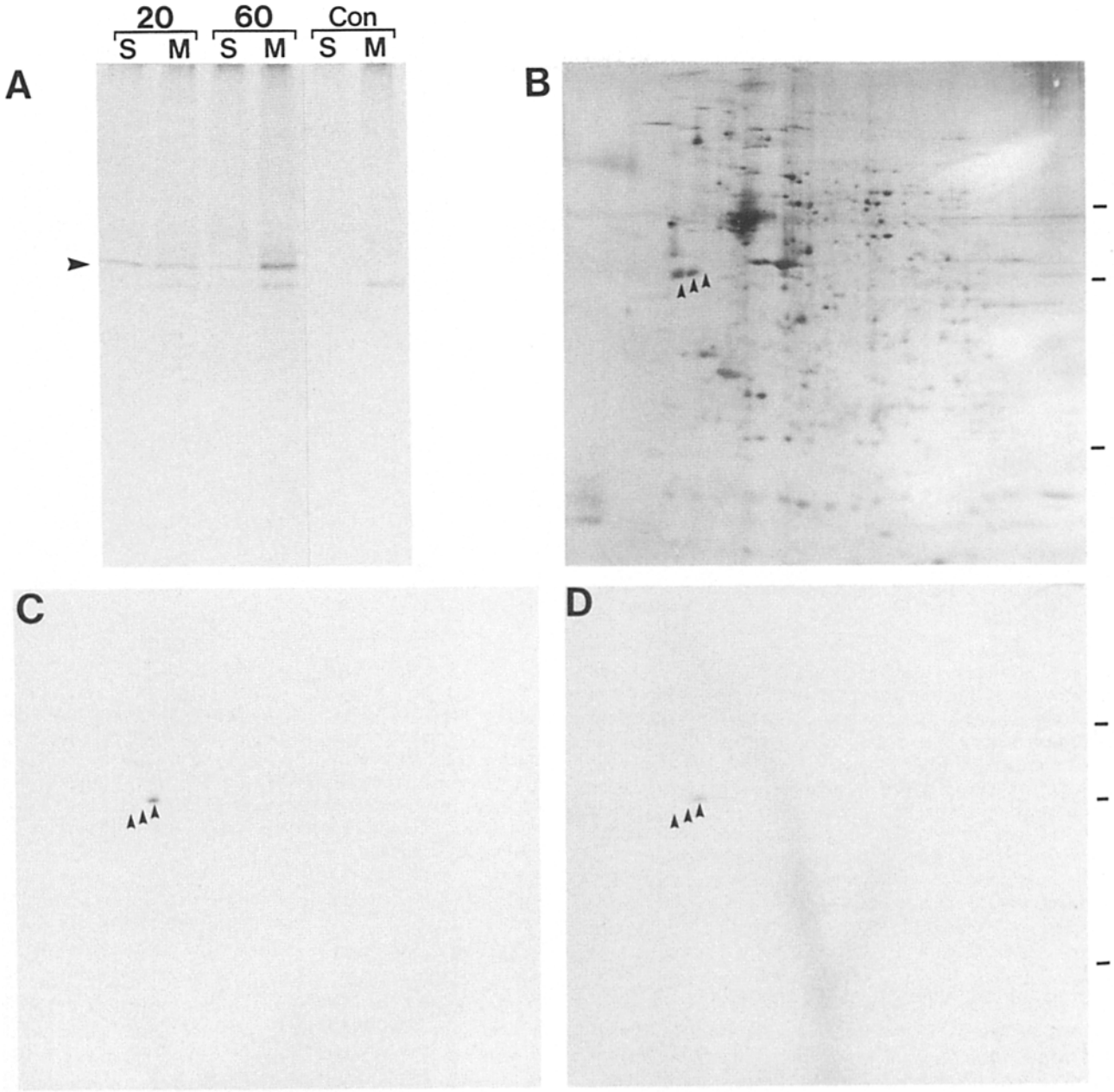

Figure 3. Identification of the initial translation product of GAP-43 in vivo. (*A*) Soluble (*S*) and membrane (*M*) fractions were prepared from 11-d-old rat cortex 20 or 60 min after intracranial injection of [^{35}S]methionine. Labeled proteins were solubilized in SDS and precipitated with antibody 91E12 (anti–GAP-43) or a control antibody (*Con*). The fraction of immunoprecipitable GAP-43 (*arrowhead*) in the soluble fraction decreases with time. (*B* and *C*) Two-dimensional gels showing the positions of the two major isoelectric forms of GAP-43 from growth cone membranes and the primary translation product of GAP-43 mRNA (*arrowheads*). The acidic end is to the left. Membranes from isolated growth cones were solubilized in SDS and mixed with the radioactive primary translation product synthesized by in vitro transcription/translation from a cloned rat GAP-43 cDNA (Basi et al., 1987). The silver-stained growth cone membrane proteins are shown in *B*; and a fluorograph of the primary translation product is shown in *C*. (*D*) Fluorograph of immunoprecipitated GAP-43 from neonatal cerebral cortex 60 min after intracranial injection of [^{35}S]methionine. The sample shown is from a membrane fraction; comigrating polypeptides were also immunoprecipitated from soluble and membrane fractions 20 min after labeling (not shown). When the samples illustrated in *C* and *D* are mixed and analyzed on the same gel, the labeled proteins produced in vivo and in vitro comigrate precisely (not shown). In these and all subsequent gels, marks at the right indicate the positions of molecular weight standards: bovine serum albumin (68,000); ovalbumin (43,000); and trypsinogen (24,500).

resolved on these gels into two major spots slightly more acidic than the primary translation product (Fig. 3). The more acidic forms of GAP-43 appear to result from phosphorylation (Zwiers et al., 1985; Schreyer, D. J., and J. H. P. Skene, unpublished observations). Association of GAP-43 with membranes precedes, and therefore does not depend upon, generation of the more acidic (probably phosphorylated) forms of the protein.

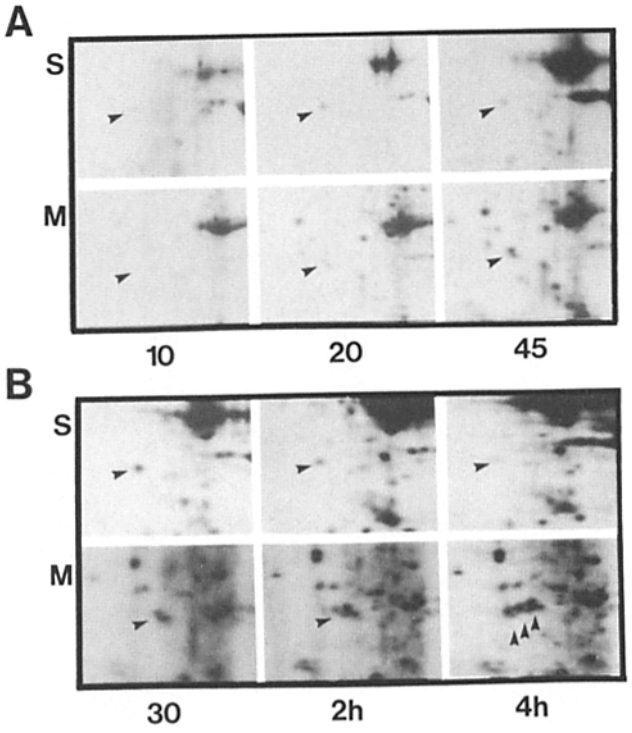

Figure 4. Transient labeling of a soluble form of GAP-43 in neonatal brain. [^{35}S]methionine was injected intracranially (*A*) or intraperitoneally (*B*) into 7-d-old rat pups. At the indicated times after injection (shown in minutes except for the 2- and 4-h time points), the cerebral cortex was removed and separated into a soluble fraction (*S*) and a crude membrane fraction (*M*). Labeled proteins were analyzed by two-dimensional electrophoresis and fluorography; the gel regions containing GAP-43 (*arrowheads*) are shown (acidic ends of IEF gels are to the left). Soluble and membrane samples for each time point are from the same animal. In the 4-h time point in *B*, the three arrowheads indicate the positions of the three isoelectric variants of GAP-43 (see Fig. 3).

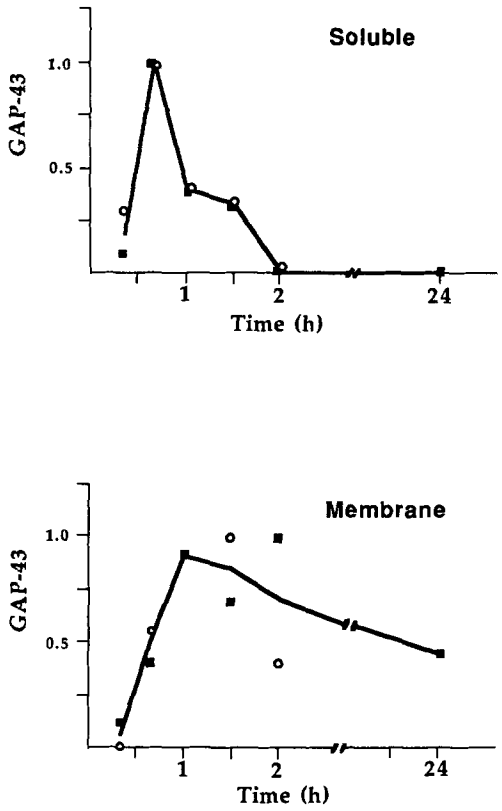

Figure 5. Labeled GAP-43 was quantitated by two-dimensional densitometry of fluorograms from two independent experiments using intraperitoneal injection of [^{35}S]methionine as illustrated in Fig. 4 *B*. Labeling was normalized to the maximum value for each series; soluble and membrane fractions were normalized separately. Maximum labeling in the membrane fractions is 10 times the maximum labeling in the soluble fractions.

Posttranslational Attachment of Fatty Acid to GAP-43

The posttranslational attachment of soluble GAP-43 to neuronal membranes raises the possibility that hydrophobic moieties involved in membrane interactions are added to GAP-43 posttranslationally. There are three well-established modifications involving covalent attachment of lipid to proteins (Sefton and Buss, 1987; Burn, 1988). The intermediate chain fatty acid myristic acid can be attached to amino-terminal glycine residues through an amide linkage (Towler et al., 1987). Longer chain fatty acids, including palmitic acid, also can be linked to proteins, generally through thioester bonds to internal cysteine residues (Kaufman et al., 1984). A more complex phosphoinositol lipid has been found to link a number of soluble proteins to cell surfaces (Low et al., 1986). The complex phosphoinositide linkage seems unlikely for GAP-43 because the protein is not exposed on neuronal cell surfaces; recent evidence also indicates that GAP-43 cannot be removed from neuronal membranes by the phosphoinositide-specific phospholipase (Perrone-Bizzozero et al., 1988). We have therefore asked whether fatty acids may be linked covalently to GAP-43.

Cultured cells from embryonic rat cerebral cortex incorporate either [^{3}H]palmitic acid or [^{3}H]myristic acid into a variety of proteins (Figs. 6 *A* and 8). As in other cells (Magee and Courtneidge, 1985; McIlhinney et al., 1985; Olson et al., 1985), myristate-labeled proteins can be detected in both the membrane and the soluble fraction, while palmitylated proteins are detected only in the membrane fraction. In primary cultures of cerebral cortical cells, [^{3}H]palmitic acid is most actively incorporated into a protein whose electrophoretic mobility is identical to mature GAP-43 (Fig. 6). Similar results were seen with the neuron-like pheochromocytoma cell line, PC12, labeled after 5 d exposure to nerve growth factor (not shown). The much smaller amount of [^{3}H]myristic acid incorporated into this protein may be attributable to partial conversion of the labeled myristic acid to longer chain fatty acids (McIlhinney et al., 1985; Olson et al., 1985). The major palmitylated protein from cortical cultures is positively identified as GAP-43 by immunoprecipitation (Fig. 6 *B*).

On two-dimensional gels (Fig. 7), [^{3}H]palmitate-labeled GAP-43 from cultured cells can be resolved into three spots, corresponding in position to the two major Coomassie Blue–stained forms of GAP-43 plus the slightly less acidic primary translation product. Fatty acid is incorporated most heavily into the most basic form, which represents a minor fraction of the total mass of GAP-43 (e.g., Fig. 3 *B*) and corresponds in position to the primary translation product (Fig. 3, *C* and

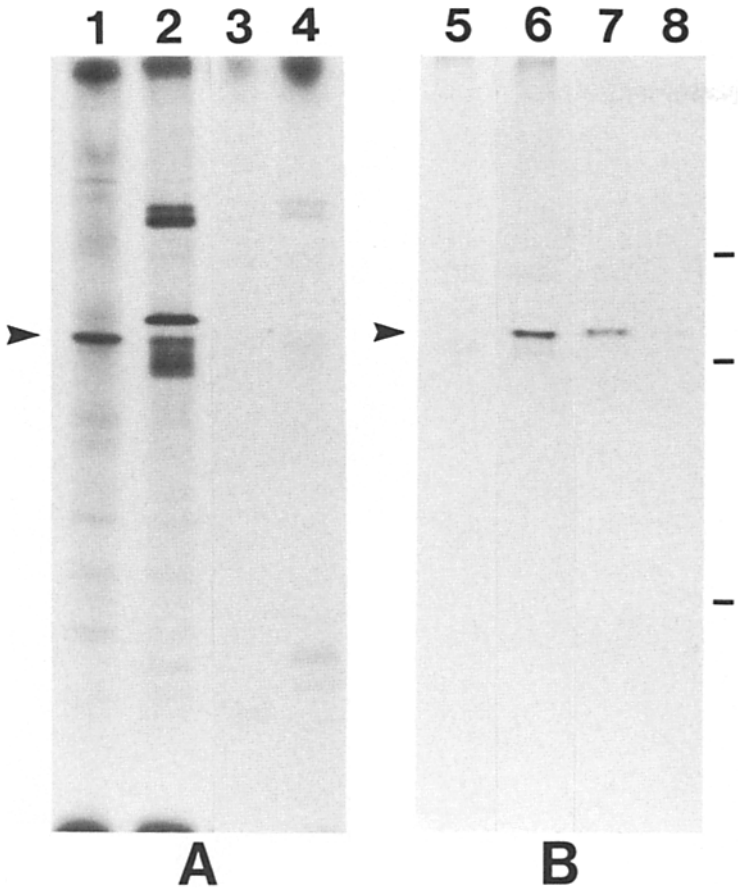

Figure 6. (*A*) Incorporation of fatty acids into proteins of intact cells from cerebral cortex. Dissociated cells from embryonic (E16) rat cortex were maintained in tissue culture for 24 h and then incubated for 2 h with either [^{3}H]palmitic acid (lanes *1* and *3*) or [^{3}H]myristic acid (lanes *2* and *4*). Cells were homogenized and separated into crude membrane (lanes *1* and *2*) and soluble (lanes *3* and *4*) fractions, and the labeled proteins analyzed by one-dimensional PAGE and fluorography. The arrowhead indicates the position of a purified GAP-43 standard electrophoresed in an adjacent gel lane. (*B*) Incorporation of [^{3}H]palmitic acid into immunoprecipitable GAP-43 by intact cells and isolated growth cones. Growth cones isolated from neonatal rat cortex (lanes *5* and *6*), and dissociated cells from embryonic rat cortex (lanes *7* and *8*), were incubated 2 h with [^{3}H]palmitic acid (lanes *5–7*) or with [^{3}H]myristic acid (lane *8*). Membrane fractions were dissolved in SDS, and GAP-43 immunoprecipitated as described under Materials and Methods. Lane *5*, control antibody; lanes *6–8*, anti-GAP-43.

D). Thus, fatty acid appears to be incorporated disproportionately into newly synthesized GAP-43. Nevertheless, the more acidic (presumed phosphorylated) forms of the protein, which appear very late in the protein's posttranslational maturation (see above), are also heavily labeled with [^{3}H]palmitic acid. It is possible that the fatty acid–labeled acidic forms of GAP-43 represent GAP-43 molecules acylated early in the 2-h labeling period and subsequently phosphorylated. Alternatively, substantial incorporation of fatty acid into the late appearing acidic forms of GAP-43 might reflect ongoing turnover of fatty acid moieties on mature GAP-43.

To determine whether fatty acid acylation of GAP-43 continues after the protein has been transported from the neuron cell body to axonal growth cones, we isolated intact growth cones from neonatal rat cerebral cortex and exposed them to [^{3}H]palmitic acid in vitro. The isolated growth cones continue to incorporate the fatty acid into membrane proteins, predominantly GAP-43 (Figs. 6 and 8). The radioactive label was not removed from GAP-43 by extensive extraction of labeled membranes with organic solvents, suggesting covalent linkage to the protein, and radioactivity recovered from labeled GAP-43 after hydrolysis in methanolic KOH comigrated on thin-layer chromatography with methyl-palmitate, indicating that the protein-bound label represents fatty acid (Fig. 8).

Provisional Assignment of Fatty Acid to cys-3 and cys-4

Palmitic acid and other long chain fatty acids are most commonly linked to proteins through thioester bonds with cysteine residues (Kaufman et al., 1984; Sefton and Buss, 1987). GAP-43 contains only two cysteine residues, adjacent to each other in a small hydrophobic domain at the amino terminus of the protein (Basi et al., 1987). To test the nature of the bond linking fatty acid to GAP-43, we exposed gels containing [^{3}H]palmitate-labeled GAP-43 to 1 M hydroxylamine at pH 7. At neutral pH, hydroxylamine hydrolyzes model compounds containing thioester bonds, but has little effect on hydroxy esters (Kaufman et al., 1984). Under these conditions, [^{3}H]palmitate was almost quantitatively removed from GAP-43 (Fig. 8).

Proteolytic mapping also indicates that the site of fatty acid attachment to GAP-43 is in the vicinity of the two cysteine residues. GAP-43 labeled with either [^{3}H]palmitic acid or [^{35}S]cysteine was isolated from growth cone membranes by two-dimensional gel electrophoresis and digested with chymotrypsin. SDS-PAGE analysis of the proteolytic products shows that both cysteine and palmitic acid are contained in a small chymotryptic fragment (Fig. 9). Sequence analysis of GAP-43 shows a single likely site for chymotrypsin hydrolysis, at phenylalanine-42 (see Basi et al., 1987 for sequence). Cleavage of GAP-43 at that site would produce a smaller (41 amino acid) peptide containing the amino terminus and a much larger (185 amino acid) peptide containing the remainder of the protein. Fig. 9 indicates the position of the large chymotryptic fragment of GAP-43, detected by Coomassie Blue staining; no radioactive fatty acid was detected in this fragment.

To determine whether the cysteine residues in GAP-43 are

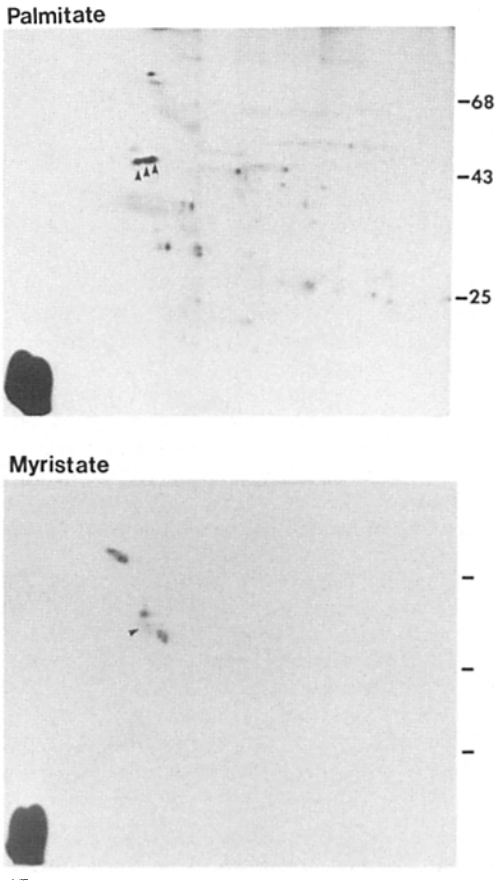

Figure 7. Two-dimensional fluorographs of embryonic cortical cells labeled with palmitic acid or myristic acid. Three arrowheads in the upper panel indicate the positions of the two isoelectric forms of GAP-43 detected by Coomassie Blue staining and the primary translation product (*rightmost arrowhead*). The arrowhead in the lower panel indicates the gel region containing Coomassie Blue–stained GAP-43.

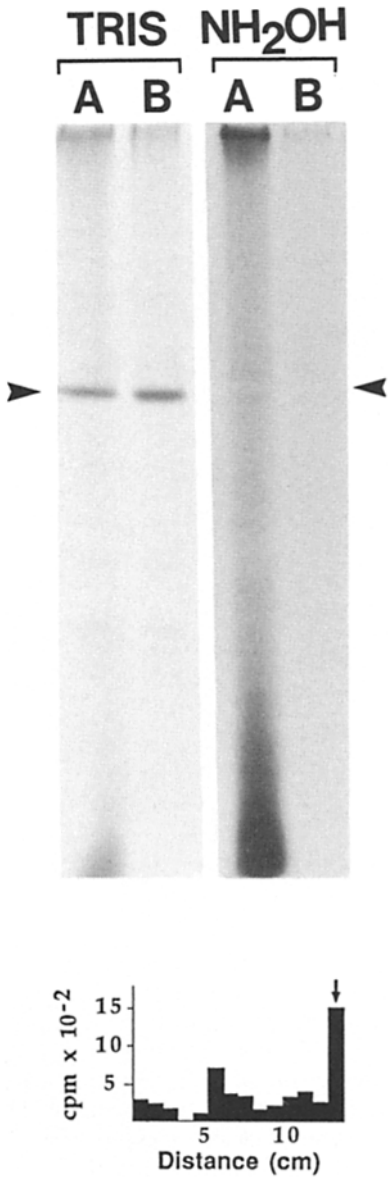

Figure 8. Analysis of the bond linking palmitic acid to GAP-43. Isolated growth cones were labeled with [^{3}H]palmitic acid as described under Materials and Methods. Growth cones were recovered by centrifugation and either resuspended directly in 1% SDS (*A*) or washed five times with chloroform/methanol (2:1) to remove noncovalently bound lipid and the dried pellet dissolved in SDS (*B*). After electrophoresis, gels were washed in 10 vol of either 1 M Tris or 1 M hydroxylamine, pH 7.0, and radioactive label remaining in the gel visualized by fluorography. The arrowheads indicate the position of Coomassie Blue–stained GAP-43 in adjacent gel lanes. The graph at bottom shows thin-layer chromatography of radioactivity recovered from palmitic acid–labeled GAP-43 after hydrolysis in methanolic KOH; the arrow indicates the position of a palmitic acid methyl ester standard.

actually involved in a thioester linkage, we used [^{14}C]iodoacetamide to probe for free thiol groups. Growth cone membranes were solubilized in SDS and pretreated for 3 h with either 1 M Tris (control) or 1 M hydroxylamine at pH 7.0. After dialysis to remove the Tris or hydroxylamine, the samples were exposed to [^{14}C]iodoacetamide in the presence of 6 M guanidine hydrochloride, to unfold proteins and ensure access of the probe to all regions of GAP-43. Fig. 10 shows that little iodoacetamide reacted with GAP-43 from control membranes, while pretreatment with neutral hydroxylamine resulted in a large increase in free thiol groups. Densitometric scanning of the autoradiographs indicates that <3% of the cysteine residues in membrane-bound GAP-43 contain free thiol groups; the remaining cysteine thiols are bound in an hydroxylamine-labile linkage. This, in turn, strongly suggests that virtually all membrane-bound GAP-43 contains fatty acid, and that fatty acid chains are attached to both cysteine residues, *cys*-3 and *cys*-4.

Discussion

Early Membrane Binding and Later Phosphorylation of GAP-43

Our observations indicate that GAP-43 is initially synthesized as a soluble protein that undergoes at least two post-translational processing events. The earlier of these, attachment of GAP-43 to neuronal membranes, seems to occur within 20 min of synthesis. 10 min after injection of label, the majority of labeled GAP-43 is soluble. Between 20 and 40 min after injection, the amount of labeled GAP-43 in the membrane fraction surpasses that in the soluble pool, despite continuing incorporation of label into soluble GAP-43 pre-

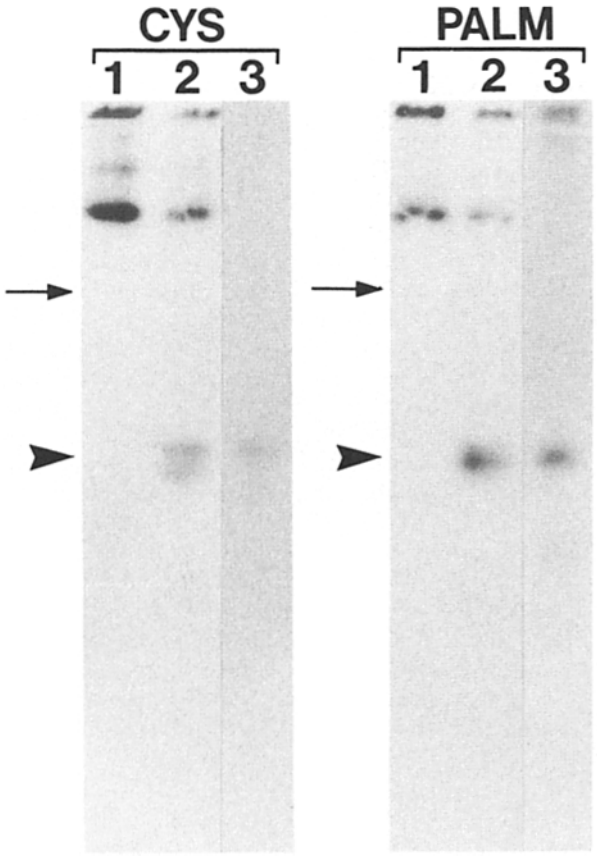

Figure 9. Chymotryptic mapping of the palmitylated domain of GAP-43. GAP-43 labeled in vivo with [^{35}S]cysteine (*CYS*) or in vitro with [^{3}H]palmitic acid (*PALM*) was isolated by two-dimensional gel electrophoresis and reelectrophoresed on a 20% acrylamide gel in the absence of protease (lanes *1*), or in the presence of 1 μg (lanes *2*) or 4 μg (lanes *3*) of chymotrypsin. Labeled peptides were detected by fluorography. A small chymotryptic peptide containing both cysteine and palmitic acid is indicated by the arrowheads. The longer arrow indicates the position of the larger GAP-43 proteolytic fragment detected by Coomassie Blue staining. In the absence of chymotrypsin, Coomassie Blue–stained intact GAP-43 comigrates with the major radioactive band.

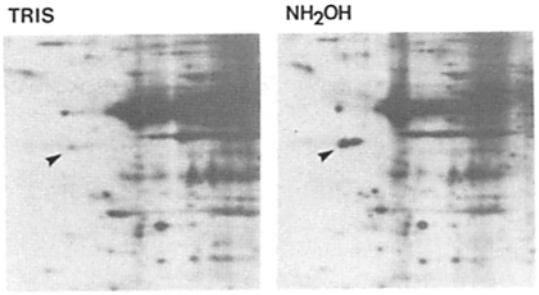

Figure 10. Iodoacetamide labeling of free cysteine residues in intact and deacylated GAP-43. Growth cone membrane proteins were denatured and incubated 3 h with either 1 M Tris, pH 7, as a control or with 1 M hydroxylamine, pH 7, to remove fatty acid bound to GAP-43. Membrane proteins were then exposed to [^{14}C]iodoacetamide to label free cysteine residues. After removal of unreacted reagents, label proteins were subjected to two-dimensional electrophoresis and visualized by autoradiography. The arrowheads indicate the position of Coomassie Blue–stained GAP-43.

cursor. It is quite possible that the attachment of GAP-43 to membranes occurs substantially <20 min after synthesis of the protein; our in vivo labeling procedures do not permit finer temporal resolution because radioactive label remains available for incorporation for 30–60 min after injection. Posttranslational transition of GAP-43 from cytosol to membranes is consistent with an earlier report that labeled GAP-43 cannot be identified in a membrane fraction from regenerating fish retina until 15–20 min after introduction of radioactive amino acid (Perrone-Bizzozero and Benowitz, 1987). The relatively rapid attachment of GAP-43 to neuronal membranes suggests that membrane attachment can occur within neuronal cell bodies, before GAP-43 is transported into axons for delivery to growth cones. This is consistent with previous reports that GAP-43 in transit along growing axons is predominantly membrane bound (Skene and Willard, 1981*c*; Benowitz and Lewis, 1983).

In contrast, a second posttranslational modification of GAP-43 does not occur for several hours after synthesis. In our study, this modification is detected as a transition of labeled GAP-43 from a single, discrete spot on two-dimensional gels to a series of spots varying in isoelectric points. At steady state (detected by Coomassie Blue or immunostaining) this heterogeneity takes the form of two major GAP-43 spots, slightly more acidic than the primary translation product (Fig. 3), and likely reflects phosphorylation of the protein by protein kinase C (Zwiers et al., 1985; Schreyer, D. J., and J. H. P. Skene, unpublished observations). The delayed appearance of charge heterogeneity in GAP-43 beginning 2–4 h after synthesis suggests that the protein is not phosphorylated until after it is transported a substantial distance along axons. GAP-43 is transported in the most rapidly moving group of axonally transported proteins, designated Group I (Skene and Willard, 1981*a*,*b*), which moves along axons at a velocity of >1 cm per hour in mammalian central nervous system axons (Willard et al., 1974; Grafstein and Forman, 1980). At that velocity, newly synthesized GAP-43 would reach growth cones or synaptic terminals of the longest intracortical axons of a neonatal rat brain by 2–4 h after synthesis. This raises the possibility that phosphorylation of GAP-43 occurs only in axon terminals.

Nature of Membrane–GAP-43 Interactions

Persistent binding of GAP-43 to growth cone membranes under a variety of ionic conditions, and strong binding of GAP-43 to detergent micelles under appropriate conditions, suggest that hydrophobic interactions play a principal role in the initial attachment of the protein to membranes and in maintaining GAP-43–membrane association. However, the highly charged primary translation product of GAP-43 mRNA appears to have no domains capable of strong hydrophobic interactions with membranes. Posttranslational addition of long chain fatty acids would provide such a hydrophobic domain. Our observations indicate that long chain fatty acids are indeed attached to GAP-43 posttranslationally, probably through thioester linkages to cysteine residues 3 and 4. When intact neurons are labeled with radioactive palmitate, the fatty acid is incorporated disproportionately (but not exclusively) into the most basic isoelectric form of GAP-43, corresponding to the initial translation product. We therefore suggest that the initial attachment of fatty acid to GAP-43 occurs shortly after the completion of translation in the neuron cell body. Fatty acylation, therefore, might be involved in the

early posttranslational association of GAP-43 with neuronal membranes.

Our observations also indicate possible modulation of GAP-43–membrane interactions by ionic conditions. Although chelation of divalent cations was not adequate to remove GAP-43 from membranes, the presence of divalent cations strongly modulates the interaction of GAP-43 with detergent micelles. This is not surprising, in light of the abundance of negatively charged residues in the sequence of GAP-43. Neutralization of some charged domains on GAP-43 by specific or nonspecific binding of cations could favor close interaction of GAP-43 with a hydrophobic environment. One important consequence of charge neutralization by divalent cations might be to permit regions of GAP-43 in addition to the fatty acid–bearing amino terminus to approach the membrane bilayer or the submembranous space. For example, as noted by Rosenthal et al. (1987), the highly negatively charged sequence in the carboxy-terminal two-thirds of GAP-43 is interrupted by a small, mildly hydrophobic region (amino acids 160–175). If the surrounding charges were neutralized by divalent cations, this region might be able to provide a second membrane-interacting domain for GAP-43.

Although we have not investigated systematically the ability of different cations to modulate the hydrophobic behavior of GAP-43, there is some indication that zinc and calcium are particularly effective. The free ion concentrations of both zinc and calcium can undergo large changes during synaptic activity, and large calcium fluxes have been reported in growth cones (Kater et al., 1988; Freeman et al., 1985; Peters et al., 1987). It will be important, therefore, to determine whether the effects of zinc and calcium on the hydrophobic behavior of GAP-43 reflect specific cation-binding sites on the protein, and whether these ions substantially modulate GAP-43–membrane interactions in growth cones and in those adult synaptic terminals that contain large amounts of GAP-43.

Relevance to Studies of Purified GAP-43

Previous work on the purification and characterization of GAP-43 has not taken into account possible effects of covalently bound fatty acid on the protein's physical and biochemical properties. Several schemes used to purify GAP-43 solubilize the protein from membranes by extracting at high pH (Zwiers et al., 1980, 1985; Chan et al., 1986; Benowitz et al., 1987). It is possible that a substantial fraction of the base-labile thioester bonds linking fatty acid to GAP-43 are hydrolyzed under these circumstances; indeed, removal of fatty acid might contribute to the removal of the protein from membranes at alkaline pH. Other purification schemes have included steps expected to enrich for hydrophilic proteins, using hydrophobic-interaction chromatography (Chan et al., 1986) or detergent-phase partitioning (Basi et al., 1987). Those procedures are likely to enrich for whatever fraction of GAP-43 lacks covalently bound fatty acid. It is interesting that both of the latter schemes yielded preparations for which the amino acid sequence began with methionine residue 5. In contrast, Storm and colleagues, using a purification scheme that included enrichment for detergent-bound protein (Masure et al., 1986), isolated a GAP-43 preparation with a blocked amino terminus, which they suggested to be acetylated (Wakim et al., 1987). After unblocking the amino terminus, those investigators determined a complete amino acid sequence, beginning with the predicted methionine-1. We suggest that Chan et al. (1986) and Basi et al. (1987) enriched for GAP-43 molecules that had lost the first four amino acids, including the fatty acylation sites at residues 3 and 4. Intact GAP-43 in those preparations would not have been detected during sequencing, because of the blocked amino terminus. In contrast, Wakim et al. (1987) detected none of the proteolyzed form of the protein, probably because it was removed during the detergent "float" used in their purification scheme. It appears that different preparations of GAP-43 may contain various proportions of molecules lacking covalently bound fatty acid, with or without the cysteine-containing amino-terminal domain of the protein.

Our own observations indicate that the majority of membrane-bound GAP-43 includes the cysteine-containing amino-terminal domain (Basi et al., 1987), and that the great majority of those cysteine residues are fatty acylated (Fig. 10). We do not know the biological relevance of the proteolytically cleaved GAP-43 isolated by Basi et al. (1987) and by Chan et al. (1986). The fact that both groups detected only one unblocked amino terminus suggests that the proteolysis is site specific and might reflect the activity of a biologically relevant protease. This raises the possibility that removal of fatty acid from GAP-43 might occur either reversibly, through cleavage of the cysteine–fatty acyl bonds, or irreversibly, through proteolytic removal of the fatty acylated amino terminus.

Dynamic Fatty Acylation of GAP-43 in Growth Cones

We suggested above that initial fatty acylation of GAP-43 occurs soon after synthesis of the protein in the neuronal cell body. Robust incorporation of labeled palmitic acid into GAP-43 in isolated growth cones strongly suggests that fatty acylation of GAP-43 continues after the protein has left the cell body and has been transported into axonal growth cones. It is important to distinguish whether ongoing incorporation of fatty acid into GAP-43 in growth cones reflects a pool of GAP-43 molecules that were transported along axons and arrived in growth cones without ever being linked to fatty acid, or whether GAP-43 molecules are actively deacylated and reacylated in growth cones. Iodoacetamide labeling of growth cone membrane proteins failed to reveal a large pool of GAP-43 molecules with free cysteine thiols. This suggests that the robust labeling of GAP-43 with palmitic acid in isolated growth cones reflects ongoing turnover of fatty acid moieties on GAP-43 molecules. A similar dynamic fatty acylation occurring hours after protein synthesis has been described recently (Magee et al., 1987) for $p21^{N\text{-}ras}$, another protein that has been suggested to modulate phosphoinositide metabolism (Fleischman et al., 1986; Wakelam et al., 1986) and to be involved in the regulation of neurite growth (Bar-Sagi and Feramisco, 1985; Hagag et al., 1986).

Because we have used only exogenously supplied palmitic acid to study fatty acylation of GAP-43, we do not know which endogenous fatty acids are most commonly attached to the protein. In other cells, the enzymes that catalyze formation of fatty acyl–protein thioesters can use a variety of longer chain fatty acids as substrates (Berger and Schmidt, 1984; Bizzozero et al., 1987). Free fatty acids available for incorporation into GAP-43 might be derived from endogenous membrane phospholipids by the actions of phospholipases A_1 and A_2, or from the receptor-mediated uptake and metabolism of extracellular lipoproteins. Developing and re-

generating nerves contain extremely high levels of lipoproteins containing apolipoprotein E (Skene and Shooter, 1983; Ignatius et al., 1986; Snipes et al., 1986), and growth cones contain an unusually high concentration of apolipoprotein E receptors. Growth cones are an especially prominent site for the receptor-mediated uptake and processing of lipoprotein-derived lipids (Ignatius et al., 1987*a*,*b*). Ongoing fatty acylation of GAP-43 in growth cones raises the possibility that modification of the protein may be modulated by transmembrane signals mediated by phospholipases and lipoprotein receptors. Fatty acylation of other proteins has been reported to be stimulated by activation of cell surface receptors (Aderem et al., 1988) or by oncogenic transformation (Burn and Burger, 1987).

Dynamic linkage of two fatty acid moieties to the extremely hydrophilic polypeptide chain of GAP-43 may allow active redistribution of the protein between membrane and cytosolic compartments, or permit regulated redistribution of the protein to appropriate domains along the lateral plane of membranes. For example, a region of membrane initially incorporated into the growth cone plasma membrane may be left behind as an axon elongates, the former growth cone membrane becoming part of the axon shaft (Pfenninger and Maylie-Pfenninger, 1981; Griffen et al., 1981; Bray and Chapman, 1985; Aletta and Greene, 1988). Deacylation might allow GAP-43 molecules to redistribute to "keep up" with the advancing growth cone or to relocate to other regions of membrane where fatty acid turnover is high.

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