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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

(axon regeneration/neuronal development/growth cone membrane proteins/antibody localization)

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ABSTRACT **Growth-associated protein, GAP-43, is a polypeptide that is induced in neurons when they grow axons. We show by means of subcellular fractionation and immunohistochemical localization that GAP-43 is a component of neuronal growth cones as well as growing neurites; it is similar to a major phosphoprotein, pp46, of a growth cone-enriched subcellular fraction. These conclusions are consistent with the possibility that the induction of GAP-43/pp46 is an important event in the establishment of a productive growth state in which a neuron is competent to extend an axon.**

A key event in axon extension, during either the initial development of the nervous system or the regenerative recovery from axon injury, is the elaboration of a growth cone—the motile structure that navigates across a tissue substrate in search of a synaptic partner, while the axon is extended behind it. Certain neuronal proteins, called growth-associated proteins (GAPs), are expressed at higher levels during periods of axon elongation (1–5); this temporal correlation has suggested that GAPs perform special functions required for axon growth, such as the formation and maintenance of a functional growth cone (2–4). We show that one of these GAPs, GAP-43, is a component of the neuronal growth cone since an antibody against GAP-43 binds to the growth cones as well as the neurites of cultured dorsal root ganglion cells. In addition, GAP-43 is a major polypeptide of a growth cone-enriched subcellular fraction (6–12) and corresponds to a phosphoprotein, designated pp46, in this fraction (7, 10). These observations show that GAP-43/pp46 is a component of growth cones and support the hypothesis that the induction of this polypeptide is a prerequisite for axon growth (1, 4).

MATERIALS AND METHODS

Preparation of Growth Cone Membranes. Growth cone membranes were prepared by subcellular fractionation in the Pfenninger laboratory, by the procedure of Pfenninger *et al.* (8, 10).

Preparation of GAP-43. Brains from 1-day-old rats or superior colliculi from 9-day-old rats were homogenized (using a motor driven glass-on-glass homogenizer) in H buffer [10 mM Tris·HCl, 2 mM dithiothreitol, 50 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (pH 7.4); 4 ml/g of tissue]. The homogenate was centrifuged at 100,000 × g_{av} for 1 hr, and the pellet was dissolved and electrophoresed, as described below.

Electrophoresis. Samples were prepared for two-dimensional electrophoresis as described (1, 2). When isoelectric focusing was used in the first dimension, the first-dimension gels were prefocused without sample for 1 hr at 200 V and then focused with sample for 8,000–10,000 V × hr. When nonequilibrium pH gradient gel electrophoresis was used in the first dimension, gels were run without prefocusing for 2700 V × hr. The $NaDodSO_4$/polyacrylamide gels that were used in the second dimensions (as well as for one-dimensional electrophoresis) contained 10% acrylamide, 0.3% bisacrylamide and the buffer systems of Laemmli (12). Gels were stained to reveal protein with either Coomassie blue (1) or silver (13).

Immunoblotting. Proteins were transferred electrophoretically from $NaDodSO_4$/polyacrylamide gels to nitrocellulose sheets by means of a modification of the procedure described by Phelps (14). $NaDodSO_4$ (0.5%) was included in the transfer buffer to transfer GAP-43/pp46 efficiently to the nitrocellulose. The antibody reaction was visualized (15) through the reaction product of horseradish peroxidase.

Preparation of Antibody. Polypeptides from a particulate fraction of the forebrains of 1-day-old rats were separated on two-dimensional gels, using nonequilibrium pH-gradient gel electrophoresis in the first dimension, and were stained with Coomassie blue. The spot corresponding to GAP-43 was cut out of the destained gel, electroeluted using the buffer system of Laemmli (12), and precipitated with a solution of chloroform and methanol (16). The back of a rabbit was injected intradermally at multiple sites with a solution containing approximately 75 μg of protein dissolved in sterile saline and emulsified with Freund's complete (first and sixth immunization) or Freund's incomplete adjuvant. The rabbit received a total of eight such immunizations at intervals of 10 days or more. Anti-GAP-43 antibody was purified from the serum by the procedure of Talian *et al.* (17); GAP-43 was transferred from one-dimensional gels to nitrocellulose; the blot was incubated with the antiserum; and then the specific anti-GAP-43 antibody was eluted from the blot.

Immunohistochemistry. Frozen sections from 17-day-old embryos, 1-day-old rats, and adult rats were prepared and immunohistochemically analyzed as described by Schwob *et al.* (18), except that sections were incubated for 0.5 hr in a solution containing 0.05% $NaDodSO_4$ in PBS [150 mM NaCl, 10 mM NaH_2PO_4 (pH 7.5)] before they were incubated with the primary antibody. This incubation with $NaDodSO_4$ increased the intensity of the subsequent immunofluorescence more effectively than similar incubation with Triton X-100. Dorsal root ganglia from 15-day-old rat embryos were grown on plastic coverslips that had been coated with collagen and

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Abbreviation: GAP, growth-associated protein.

air dried (19). Two-day-old cultures were fixed for immunofluorescence with either methanol at −20° or 4% (wt/vol) paraformaldehyde. Subsequent treatment, including incubation with $NaDodSO_4$, was the same as for frozen sections.

Protein Determinations. Protein was measured by the method of Bradford (20).

RESULTS

Anti-GAP-43 Binds to Growth Cones and Neurites. To investigate the disposition of GAP-43 within neurons, we prepared an antibody that reacts specifically with GAP-43, as judged by its ability to bind specifically to a spot of the appropriate two-dimensional electrophoretic mobility on an immunoblot (see Fig. 4). Because biochemical evidence has indicated that GAP-43 is approximately 10-fold more concentrated in developing nervous tissue than adult nervous tissue, the antibody was used first to label frozen sections of nervous tissue from rats of different ages (17-day-old embryos, 1-day-old rats, and adult rats; supplied by J. Schwob) by means of indirect immunofluorescence. Brain tissue from the embryonic and neonatal animals bound the anti-GAP-43 extensively (Fig. 1). Areas of the central nervous system [e.g., the optic fiber layer of the retina and certain axon bundles in the cortex (not shown)] were intensely fluorescent. In addition, peripheral cutaneous nerves (not shown), the olfactory tract (not shown), and fibers in the olfactory epithelium (not shown) were highly labeled. The antibody did not react with non-neuronal tissue, such as the bones, connective tissue, and skin, in these whole head mounts.

In contrast, when frozen sections of adult rat brain were labeled and photographed under identical conditions, the general intensity of fluorescence was much lower than in the developing animals (Fig. 1). Under more optimal conditions of exposure, certain areas—such as the peripheral trigeminal nerve—appeared less fluorescent than the surrounding areas of the section. On the other hand, the dentate gyrus reacted more intensely than the surrounding tissue, suggesting that it continues to express relatively elevated levels of GAP-43 in the adult (not shown). These results confirm that more antibody against GAP-43 should bind to developing nervous tissue than to adult nervous tissue.

The anti-GAP-43 antibody was then used to localize GAP-43 in 2-day-old cultured dorsal root ganglia explants from 15-day-old rat embryos (supplied by N. Ratner). The outer circumference of the halo of neurites, which is the location of the growing tips, was the most intensely labeled area (Fig. 2*a*). Observation at high magnification showed that this circumferential fluorescence was associated with growth cones (Fig. 2 *e* and *f*). In addition, the neurites fluoresced in a discontinuous, punctate pattern. The presence of labeled growth cones and neurites was independent of whether the cultures were fixed with cold methanol or paraformaldehyde (Fig. 2*d*). Preliminary observations indicate that the antibody will also react with cultures that have been fixed but have not been treated with detergent. We conclude that, in cultured dorsal root ganglion cells, GAP-43 is a component of the growth cone and is also present in the neurites.

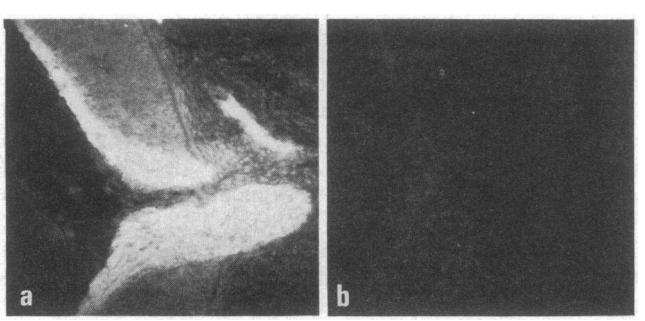

FIG. 1. Antibody-mediated fluorescent labeling of tissue with anti-GAP-43. (*a*) Optic fiber layer of retina from 17-day-old embryonic rats, labeled with anti-GAP-43. (*b*) Optic fiber layer from adult rats labeled with anti-GAP-43 and photographed under conditions identical to those used in *a*. Magnification is 30×.

Electrophoretic and Antigenic Similarities Between GAP-43 and pp46. Pfenninger *et al.* (6–11) have developed a procedure for purifying growth cone particles from embryonic brain tissue. Fig. 3 shows that, a major polypeptide of this growth cone-membrane fraction, designated pp46 (Fig. 3*a*), has the same electrophoretic mobility as GAP-43 (Fig. 3*c*) on two-dimensional polyacrylamide gels, when either isoelectric focusing or nonequilibrium pH-gradient gel electrophoresis (not shown) was employed as the first dimension. For these experiments, the source of GAP-43 was a membrane fraction from the superior colliculus of 9-day-old rats. The silver-stained polypeptide designated GAP-43 was identified as such by virtue of its comigration on two-dimensional gels with radiolabeled, axonally transported GAP-43 (not shown). In addition, like labeled GAP-43, pp46 composes a 10- to 20-times greater portion of the total membrane protein in neonatal animals than in adults (not shown).

As an additional assay for the similarity of GAP-43 and pp46, we used immunoblotting to compare their reactivity with the affinity-purified antibody prepared against GAP-43. Fig. 4 shows that this antibody reacted specifically with GAP-43 (from a particulate fraction of neonatal rat brain) and with pp46 (from the growth cone membrane fraction) when the proteins from these sources were separated by two-dimensional gel electrophoresis, transferred to a nitrocellulose sheet, and indirectly labeled with the antibody. We conclude that the growth cone subcellular fraction contains a major polypeptide (pp46) with antigenic and electrophoretic properties that are indistinguishable from GAP-43 and that both polypeptides react with an antibody against GAP-43.

GAP-43/pp46 Is Enriched in the Growth Cone-Membrane Subcellular Fraction. If GAP-43/pp46 were preferentially associated with growth cones, as suggested by the immunofluorescence experiments, it should be most enriched in the subcellular fractions that contain the purest population of growth cones. The partial purification of growth cone membranes from the forebrains of 17-day-old rat embryos by the procedure of Pfenninger *et al.* (8, 10) involves the following steps: (*i*) homogenization of the tissue; (*ii*) low-speed centrifugation; (*iii*) fractionation of the supernatant on a three-step discontinuous sucrose density gradient, whose fractions are designated A, B, and C in order of increasing density (fraction A is most enriched in particles that morphologically resemble growth cones); (*iv*) preparation of membranes from this growth cone fraction. We qualitatively estimated the relative enrichment of GAP-43/pp46 in the different subcellular fractions by electrophoresing equal amounts of total protein from each fraction on one-dimensional gels and observing the intensity of reaction between anti-GAP-43 and the GAP-43 from each fraction by means of immunoblots (Fig. 5). The reaction was most intense in the growth cone membrane fraction (Fig. 5*g*) and in the low-density sucrose gradient fraction from which these membranes were derived (fraction A, Fig. 5*f*). However, fraction B from the sucrose gradient (Fig. 5*e*), which typically contains a morphologically heterogeneous mixture of elements (8), also reacted strongly with the anti-GAP-43 antibody.

To obtain a more quantitative estimate of the relative enrichment of GAP-43/pp46 in these subcellular fractions, we first electrophoresed aliquots of each fraction on two-dimensional gels to determine the volume required to generate the same degree of silver staining of the electrophoretic

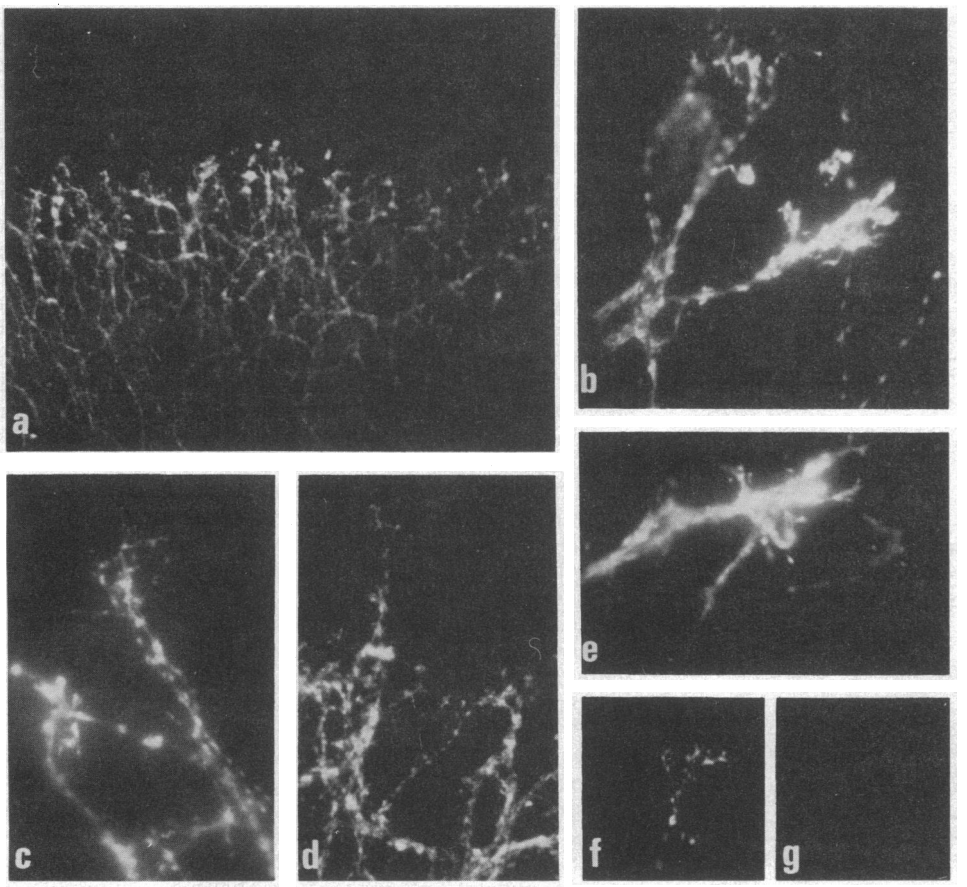

FIG. 2. Antibody-mediated fluorescent labeling of 2-day-old cultures of explants from 15-day-old embryonic rat dorsal root ganglia with anti-GAP-43 antibody. (*a*) Circumferential labeling of cultures. (24×.) (*b*, *c*, and *d*) Punctate labeling of neurites. (60×.) (*e* and *f*) Higher magnification (95×) of growth cones. (*g*) Control culture incubated with nonimmune IgG. (60×.) Cultures were fixed with methanol at −20°C before they were incubated with the antibody, except for (*d*), which was fixed with 4% (wt/vol) paraformaldehyde.

spot corresponding to GAP-43/pp46. The degree of staining was quantitated by densitometric scanning; the similarity of staining in different fractions was also apparent from inspection (Fig. 6). The amount of total protein in these volumes (which contained approximately equal amounts of GAP-43/pp46) provides an estimate of the relative enrichment of GAP-43/pp46. The results (Fig. 6) show that GAP-43/pp46 is most enriched in the growth cone membrane fraction, where it constitutes a 10-fold greater proportion of the total protein than in the initial homogenate. (The enrichment of GAP-43/pp46 in the growth cone membrane fraction from two separate preparations that we analyzed was 15- and 30-fold compared to the original homogenate.) Fractions A and B from the sucrose gradient were also enriched substantially, by factors of 5 and 7, respectively, compared to the original homogenate. Fig. 6 also serves to illustrate the fates of various polypeptides during the course of the growth cone purification.

DISCUSSION

The ability of the growth cone to perform the specialized functions of directed motility must depend on its particular molecular composition. If GAP-43 were an essential component of the growth cone, then its induction could be one of the critical steps in converting a neuron to a metabolic state in which it is competent to extend an axon (1, 2, 4). In this case, the failure of a neuron to raise its level of GAP-43 after axon injury—as has been observed in certain neurons of the

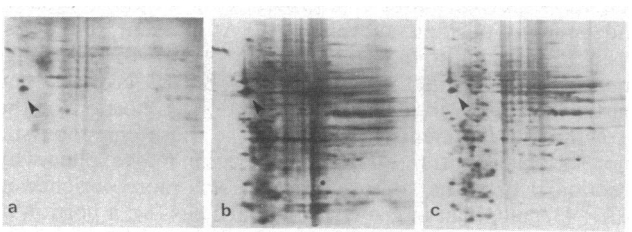

FIG. 3. Comparison of the electrophoretic mobilities of pp46 (*a*; from the growth cone-membrane subcellular fraction of a 17-day-old embryonic rat) with GAP-43 (*c*; from the superior colliculus of a 9-day-old rat) on silver-stained two-dimensional gels, with isoelectric focusing in the first dimension. Approximately 4.7 and 55 μg of protein were applied to gels (*a*) and (*c*), respectively. When these same amounts were mixed and electrophoresed on the same gel (*b*), GAP-43 and pp46 electrophoresed to the same location. The arrows indicate the position of GAP-43/pp46, which corresponds to a 45-kDa polypeptide with a pI of 4.3. The pH gradient is, from left to right, pH 4.0 to 8.5.

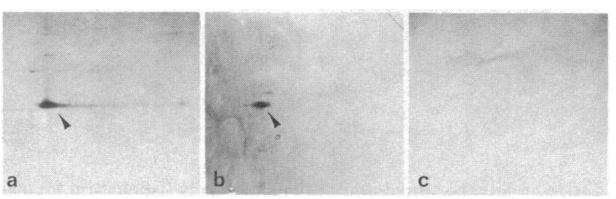

FIG. 4. Reaction of anti-GAP-43 antibody with GAP-43 (*a*; from a particulate fraction from 1-day-old rat brain) and pp46 (*b*; from the growth cone-membrane subcellular fraction). The polypeptides from these fractions were separated by two-dimensional electrophoresis, with nonequilibrium pH-gradient gel electrophoresis in the first dimension. The polypeptides were transferred to a sheet of nitrocellulose and reacted with the antibody (immunoblotted). Approximately 75 μg (*a*) and 15 μg (*b*) of protein was electrophoresed, and the affinity-purified antibody was used at a concentration of 0.2 μg/ml. (*c*) Nonimmune IgG (0.2 μg/ml) did not label any band on a blot that was otherwise identical to *a*. In each figure the acidic side of the gel is on the left.

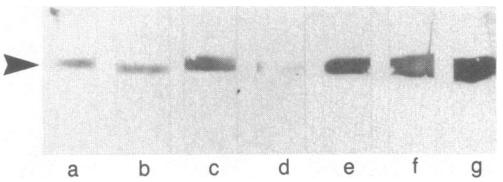

FIG. 5. Reaction of anti-GAP-43 antibody with GAP-43/pp46 from different subcellular fractions of the growth cone preparation (12). Equal amounts (35 μg) of protein from each of the following fractions were electrophoresed on one-dimensional gels, transferred to nitrocellulose, and incubated with the anti-GAP-43 antibody at a concentration of approximately 0.2 μg/ml. (*a*) Initial homogenate. (*b* and *c*) Pellet and supernatant, respectively, of initial homogenate. (*d*, *e*, and *f*) Fractions C, B, and A from the sucrose gradient (fraction A is most enriched in growth cone particles; refs. 8 and 10). (*g*) Membranes prepared from the growth cone particle fraction A. The arrow indicates the position of GAP-43.

mammalian central nervous system (1)—could limit its ability to regenerate an axon. These considerations raise the question, addressed here, of whether GAP-43 is associated with growth cones. The results show that GAP-43 is indeed a component of the growth cone membrane. First, immunohistochemical experiments showed that an antibody against GAP-43 bound to growth cones (and also neurites) of cultured dorsal root ganglia. Second, GAP-43 was enriched in the membranes from a subcellular fraction that is most enriched in organelles that appear to be derived from neuronal growth cones. GAP-43 is similar, if not identical, to a major polypeptide of this subcellular fraction, designated pp46 (7, 10). The observed association of GAP-43/pp46 with growth cones fulfills one requirement of the hypothesis that the induction of GAP-43/pp46 serves to regulate axon growth by supplying a protein that is essential to the function of the growth cone.

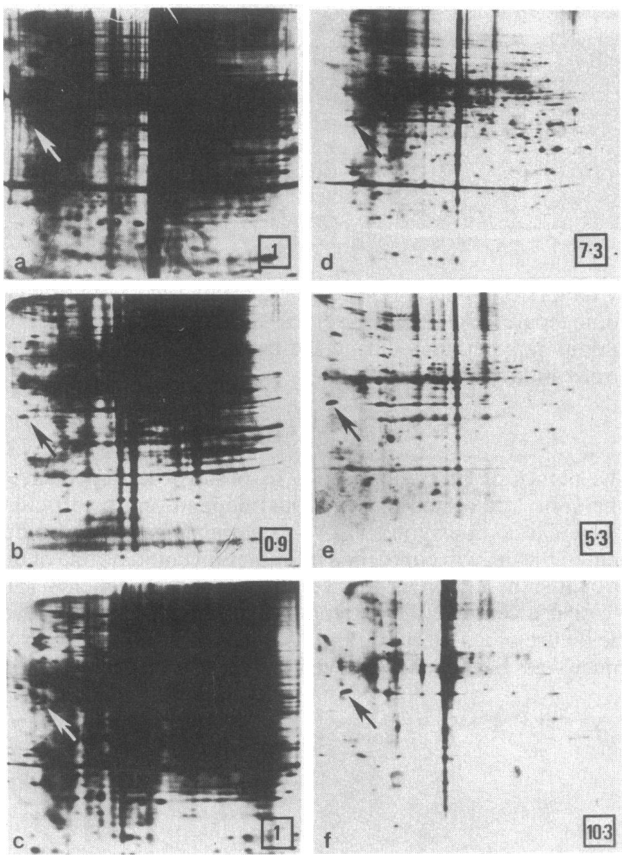

FIG. 6. Silver-stained two-dimensional gels (isoelectric focusing in the first dimension) showing polypeptides of the subcellular fractions from the growth cone preparation. Each gel contains similar amounts (variation between extremes is 12%) of GAP-43/pp46, judged by densitometric scanning of the gels. The relative enrichment of each fraction with respect to GAP-43 (number in each panel) was calculated as the reciprocal of the amount of total protein applied to the gel and normalized to the amount of initial homogenate. The fractions are as follows: initial homogenate (*a*); supernatant of the low speed centrifugation of the initial homogenate (*b*); fractions C, B, and A from the sucrose density gradient (A, the least dense fraction, is most enriched in growth cone particles) (*c*, *d*, and *e*); membranes prepared from the growth cone particle fraction A (*f*). The pH gradient is the same as in Fig. 3.

To evaluate this hypothesis further, it would be useful to know whether the function of GAP-43 is unique to growth cones (as would be suggested if it were preferentially localized in growth cones) or, alternatively, whether it performs a more general function throughout the plasma membrane. The current observations do not resolve this question. It is clear from the immunohistochemical experiments that GAP-43 antigens are present in neurites as well as growth cones of dorsal root ganglion cell explants. In these experiments, the growth cones bound more anti-GAP-43 antibody than the neurites, suggesting that GAP-43/pp46 is more concentrated in the growth cone. However, the observed difference in the intensity of the immunofluorescence reaction could reflect differences in the geometry or in the accessibility of the antibody as well as differences in the concentration of GAP-43/pp46 in the two regions of the cell. In the regenerating toad visual system, the distribution of biosynthetically radiolabeled GAP-43 along the retinal ganglion cell axons is consistent with the interpretation that it is most concentrated in the growing tips (3). One possibility is that GAP-43/pp46 functions primarily in the growth cone and that the characteristic punctate staining of the neurites represents the compartments within the neurite in which GAP-43/pp46 is conveyed, by the process of rapid axonal transport, from the cell body to its functional destination. Although the estimated half-life of GAP-43 (several days) during regeneration of the toad optic nerve (3) suggests that only a small fraction of the GAP-43 in the growth cone would be in rapid transit down the axon at a particular time to maintain the supply of GAP-43 to the growth cone, we do not know whether a similar half-life of GAP-43/pp46 applies to these neurons in culture.

The association between GAP-43/pp46 and growth cones is also indicated by its enrichment (10- to 30-fold) in the subcellular fraction in which more than 70% of the structures appear to be derived from growth cones (8). Although the specificity of the association between GAP-43/pp46 and growth cones could be evaluated by comparing their enrichment in each subcellular fraction, such an analysis would require a specific marker for growth cone fragments that is independent of their morphology. At present, the most specific marker for the identification of growth cone fragments is the distinctive morphology of the growth cone particles. However, during fractionation, a portion of the growth cone fragments is likely to lose these characteristic morphological features. This consideration may explain why fraction B from the sucrose gradient was more enriched in GAP-43/pp46 than in growth cone particles (8); a portion of the GAP-43/pp46 in fraction B may have been associated with growth cone fragments that were morphologically unrecognizable. However, this differential enrichment of GAP-43/pp46 in fraction B may also reflect an association between GAP-43/pp46 and other structures, such as axon segments, which may be concentrated in fraction B. Hence, the question of the degree of specificity of the association between GAP-43 and growth cones remains open.

The observation that pp46—a major polypeptide of the growth cone particle fraction—reacts with an antibody that also reacts with growth cones, is consistent with the consid-

erable evidence that the major component of this subcellular fraction is indeed derived from growth cones (8). Similarly, a monoclonal antibody against another polypeptide of the growth cone particle fraction, designated 5B4 (10), also reacts with growth cones and neurites of cultured neurons. Like GAP-43/pp46, 5B4 is expressed at elevated levels in the developing nervous system (11).

GAP-43 is very likely the same protein as pp46. The two polypeptides behaved indistinguishably in three electrophoretic systems (isoelectric focusing, nonequilibrium pH-gradient gel electrophoresis, and $NaDodSO_4$/polyacrylamide gel electrophoresis), indicating that their isoelectric points (pI = 4.3), apparent molecular size (45 kDa), and charge are similar. The difference in the reported molecular sizes of GAP-43 and pp46 (43 kDa as compared to 46 kDa, respectively) is a consequence of variations between species (toad and rabbit compared to rat) and also of the unusual way in which the electrophoretic mobilities of these polypeptides depend upon the conditions of electrophoresis (e.g., the concentration of acrylamide; ref. 21). In addition, both polypeptides reacted with the antibody prepared against GAP-43. Furthermore, previous independent characterization of these polypeptides has revealed the following similarities: both pp46 (7) and GAP-43 (4) are phosphoproteins, whose phosphorylation *in vitro* is stimulated by calcium and calmodulin as well as by phosphatidylserine; both are integral plasma membrane proteins associated with the most rapidly axonally transported group of polypeptides (group I; refs. 1, 2, and 9); and both are expressed at increased levels in neurons with growing axons (1, 2, 9). It is, therefore, highly likely that the two polypeptides are identical.

It has become apparent that, in addition to their similarity to each other, GAP-43 and pp46 are very similar to two other proteins, F1 (22–24) and B50 (ref. 25 and unpublished results), which have been studied primarily in the adult nervous system. We observed here that the anti-GAP-43 antibody reacted weakly but significantly with adult brain tissue. These observations and biochemical measurements that indicate that GAP-43/pp46 is present in adult nervous tissue (albeit at levels lower by factors of 10 to 20 than in developing nervous tissue; refs. 1 and 7) suggest that GAP-43/pp46 also performs a function within the mature neuron. F1 has been reported to be phosphorylated in a manner that is correlated with the acquisition of longterm posttetanic potentiation (22, 23), a plastic change in synaptic efficacy that has been used as a model system to study mechanisms of memory. It is interesting to consider that these proteins may perform functions that are necessary for certain forms of neuronal plasticity (e.g., longterm posttetanic potentiation) in the adult nervous system and that these functions are similar to those that are required to a much greater degree in the plastic process of growth cone-mediated axon extension.

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