Tubulin Is Phosphorylated at Tyrosine by pp60<sup>src</sup> in Nerve Growth Cone Membranes

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Abstract. We show here that tubulin is the major in vivo substrate of the tyrosine-specific protein kinase pp60<sup>src</sup> in nerve growth cone membranes. Phosphotyrosine antibodies were used to demonstrate phosphotyrosyl residues in a subpopulation of α- and β-tubulin that was highly enriched in a subcellular fraction of growth cone membranes from fetal rat brain. The presence of phosphotyrosine-modified isoforms of α- and β-tubulin in vivo was confirmed by 32P labeling of rat cortical neurons in culture. Tubulin in growth cone membranes was phosphorylated at tyrosine in endogenous membrane phosphorylation reactions (0.068 mol phosphotyrosine/mol α-tubulin and 0.045 mol phosphotyrosine/mol β-tubulin), and phosphorylation was specifically inhibited by antibodies directed against pp60<sup>src</sup>, which is localized in the growth cone membranes. pp60<sup>src</sup> was capable of directly phosphorylating tubulin as shown in immune complex kinase assays with purified brain tubulin. Phosphopeptide mapping revealed a limited number of sites of tyrosine phosphorylation in α- and β-tubulin, with similar phosphopeptides observed in vivo and in vitro. These results reveal a novel posttranslational modification of tubulin that could regulate microtubule dynamics at the growth cone.

The nerve growth cone is a structure rich in cytoskeletal elements that is located at the distal tip of outgrowing neuronal processes (neurites). Locomotion, pathfinding, and recognition of synaptic targets are growth cone-mediated functions that are essential to the development of the nervous system. These functions are achieved by coordinated assembly of microtubules in concert with actin filaments and neurofilaments, resulting in directional outgrowth of neurites (Mitchison and Kirsch, 1988). The mechanism by which the growth cone navigates through the developing tissue is not understood, but is likely to involve transduction of extracellular guidance cues, from cell adhesion molecules and extracellular matrix (reviewed in Jessell, 1988), into intracellular signals (Schuch et al., 1989).

Tyrosine-specific protein kinases represent a class of signal-transducing enzymes with established roles in regulating cell proliferation and differentiation (Hunter and Cooper, 1984; Yarden and Ullrich, 1988). The src family of protooncogenes encodes nonreceptor tyrosine kinases that are located at the inner surface of the plasma membrane. The prototype of this family is the normal cellular src gene, whose product, pp60<sup>src</sup>, is a tyrosine kinase (Collett and Erikson, 1978; Hunter and Sefton, 1980) that is preferentially expressed in embryonic neural tissues, but whose function has yet to be elucidated (Cotton and Brugge, 1983; Levy et al., 1984). A role for pp60<sup>src</sup> action in the growth cone is indicated from our recent finding that pp60<sup>src</sup> is highly enriched in nerve growth cone membrane preparations (Maness et al., 1988). This localization is consistent with the onset of pp60<sup>src</sup> expression in terminally differentiating neurons when cell division ceases and neurites are extended (Sorge et al., 1984; Fults et al., 1985). A specialized role for pp60<sup>src</sup> in neuronal cells is further underlined by the presence of a unique, more active form of pp60<sup>src</sup> (Brugge et al., 1985; Bolen et al., 1985; Matten and Maness, 1987) that is the product of alternatively spliced mRNA in neurons and certain neuroblastoma cells (Levy et al., 1987; Martinez et al., 1987). This high specific activity form of pp60<sup>src</sup> is the predominant species in growth cone membranes (Maness et al., 1988).

The identification of intracellular targets of pp60<sup>src</sup> action would provide a key to understanding its role in nerve growth cones. Here we report that a subpopulation of tubulin is the principal substrate of pp60<sup>src</sup> in growth cone-enriched membranes from fetal rat brain.

Materials and Methods

Antibodies

Polyclonal antibodies specific for protein phosphotyrosyl residues were raised against the autophosphorylated v-Ab1 protein expressed in bacteria and affinity purified on phosphotyrosine-Sepharose (Wang, 1985; Wang and Baltimore, 1985). Monoclonal antibodies specific for phosphotyrosyl (PY20) (Glenney et al., 1988), α-tubulin (DM-1A), and β-tubulin (DM-1B) were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Monoclonal antibodies specific for β-tubulin (TUB 2.1) and polyclonal antibodies that
recognize both α- and β-tubulin were from Sigma Chemical Co. (St. Louis, MO). Antibody YL1/2 was obtained from Sera-Lab (Westbury, NY). mAbs that recognize mammalian pp60 src were mAb327 (Lipschitz et al., 1983; provided by J. Brugge [University of Pennsylvania, Philadelphia, PA]) and mAb GD11 (Parsons et al., 1986; Oncor, Inc., Gaithersburg, MD). Monoclonal antibodies against S100 protein were the gift of Y. Gillespie (University of Alabama, Birmingham, AL).

**Subcellular Fractionation of Fetal Rat Brain**

Brains of fetal Sprague-Dawley rats of 18-d gestational age (Charles River Breeding Laboratories, Inc., Boston, MA) were fractionated by a modification (Maness et al., 1988) of the method of Pfenniger et al. (1983). Briefly, brain homogenates were centrifuged at 1,660 g to produce a low-speed supernatant, which was subjected to centrifugation through a three-step discontinuous sucrose density gradient to yield the A-fraction at the 0.32 M/0.75 M sucrose interface. This fraction contains at least 70% axonal growth cone particles identified by electron microscopy (Pfenniger et al., 1983) and protein profile (Simkowitz et al., 1989). The B-fraction, obtained from the 0.75 M/1.0 M sucrose interface, contained lysed growth cones, small neuronal and glial fragments, and Golgi cisternae. The C-fraction, obtained from the 1.0 M/2.66 M sucrose interface, contained neuritic shafts, rough endoplasmic reticulum, and mitochondria. Salt-washed membranes from each fraction were prepared by hypotonic lysis in 6 mM Tris- HCl, 0.5 mM Na-EDTA (pH 8.3); washing in 0.3 M NaNO3, 20 mM Hepes (pH 7.4) then in 20 mM Tris- HCl, 10 mM β-mercaptoethanol, 1 mM MgCl2, 1 mM Na-EGTA, 1 μg/ml pepstatin A (pH 7.4); and resuspension in 20 mM Tris- HCl, 1 mM MgCl2, 1 μg/ml pepstatin A (pH 7.4). Buffers were supplemented with 10 mM NaF and 200 μM Na3VO4 to inhibit tyrosine phosphatases, and aprotinin (100 KIU/ml) and 0.1% leupeptin to prevent proteolysis. Protein was measured by the method of Lowry et al. (1951).
Figure 1. Endogenous phosphorylation of tubulin in growth cone–enriched membranes. (A) Silver-stained proteins in A-fraction membranes (30 μg) separated by two-dimensional gel electrophoresis; 2% ampholines (pH 3–10). (B) Proteins phosphorylated by endogenous phosphorylation in A-fraction membranes (30 μg), separated by two-dimensional gel electrophoresis (1.5% ampholines [pH 4–6], 0.5% ampholines [pH 3–10]), and transferred to an Immobilon filter. (C) Immunostaining of the filter in B with monoclonal antibodies specific for α- (DM-1A) and β-tubulin (TUB 2.1) and secondary antibodies conjugated to horseradish peroxidase. Arrows in B were placed to mark the limits of tubulin immunostaining in C. Internal protein standards were (α) phosphorylase b (pI 5.6–5.8); (γ) BSA (pI 4.7); (γ) ovalbumin (pI 4.6). Autoradiography was for 90 min.

1987). Immune complexes were incubated for 40 min at 30°C in a reaction mixture containing 0.05 M Pipes (pH 6.9), 20 μM Na-ATP, 40 μCi [γ-32p] ATP (17 Ci/mmol), 5 mM MnCl2, 1 μM protein kinase A inhibitor peptide, 50 μM Na3VO4, 100 KIU/ml aprotinin, 0.01% (wt/vol) leupeptin, and unassembled tubulin (2 μg) in a final volume of 32 μl. In some experiments, A-fraction membranes (30 μg) were substituted for pp60Eng immune complexes. The reaction was terminated by the addition of 15 mM Na-EDTA, 6 mM Na-GTP, and the solution incubated at 4°C for 20 min before the removal of the immune complexes or membranes by centrifugation. For analysis of stoichiometry, α- and β-tubulin subunits were separated by two-dimensional gel electrophoresis, excised from dried gels and counted for 32P in Aquasol (New England Nuclear, Boston, MA) in a liquid scintillation counter.

**Phosphoamino Acid Analysis**

For phosphoamino acid analysis, acid hydrolysis was performed directly on excised pieces of Immobilon filters (Kamps and Seflon, 1988) or on proteins excised from dried gels (Cooper et al., 1983). The recovered phosphoamino acids were subjected to two-dimensional separation on cellulose-coated thin layer sheets by electrophoresis at pH 1.9, followed by ascending chromatography in 5:3 isobutyric acid/0.5 M ammonium hydroxide (vol/vol) (Cooper et al., 1983).

**Results**

**Endogenous Tyrosine Phosphorylation of Tubulin in Growth Cone Membranes In Vitro**

Membranes from the growth cone–enriched A-fraction from fetal rat brain contained approximately 50 polypeptides resolved by two-dimensional gel electrophoresis (Fig. 1 A). An enrichment of proteins of 55–53, 46, 42, and 34 kD, and relatively low amounts of high molecular mass proteins in A-fraction membranes reflects the simpler pattern of polypeptides in this fraction as reported previously (Simkowitz et al., 1989). High molecular mass glycoproteins are present in this fraction, but migrate more diffusely (Ellis et al., 1985; Maness et al., 1988). The two major proteins at 55–53 kD co-migrated with purified α- and β-tubulin, and together accounted for ∼5% of the total membrane protein. Tubulin was tightly associated with A-fraction membranes, as it was not extracted by 0.3 M Na2SO4 or saponin (Simkowitz et
accounted for 82% of the $^{32}$P-labeled phosphoamino acids in the protein colocalizing with $\alpha$-tubulin, and 80% of the phosphoamino acids in the protein colocalizing with $\beta$-tubulin, as determined by scintillation counting. Prolonged autoradiographic exposure revealed phosphothreonine, in addition to phosphotyrosine and phosphoserine.

The extent of phosphorylation of the proteins co-migrating with $\alpha$- and $\beta$-tubulin was determined after endogenous phosphorylation in A-fraction membranes under conditions at which maximal levels of tyrosine phosphorylation were reached in both subunits (40 $\mu$M ATP, 30°C, 40 min). The phosphorylation of tubulin at tyrosyl residues, as determined from $^{32}$P incorporation, was 0.068 mol phosphotyrosine/mol $\alpha$-tubulin and 0.045 mol phosphotyrosine/mol $\beta$-tubulin. The number of tyrosyl residues phosphorylated in

**Figure 2.** Phosphoamino acid analysis of tubulin phosphorylated in growth cone membranes or purified tubulin phosphorylated by pp60$^{src}$. (A) $\alpha$- and $\beta$-tubulin in growth cone membranes (15 $\mu$g) were $^{32}$P labeled in a membrane phosphorylation reaction, resolved by SDS gel electrophoresis, transferred to Immobilon, and analyzed for phosphoamino acid content. (B) Bovine brain tubulin (8 $\mu$g) was phosphorylated by pp60$^{src}$ in the immune complex kinase assay, and $\alpha$- and $\beta$-tubulin were separated by one-dimensional SDS gel, excised from the dried gel, and analyzed for phosphoamino acid content. The positions of internal phosphoamino acid standards were revealed by ninhydrin staining and are circled. Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine.

al., 1989). The heterogeneity of $\alpha$- and $\beta$-tubulin in the isoelectric focusing dimension was indicative of multiple isoforms which have been demonstrated in brain (Binet and Meininger, 1988) and may carry out distinct cellular functions (Joshi and Cleveland, 1989).

A-fraction membranes were incubated with $[\gamma^{32}]$P]ATP in an endogenous protein kinase assay in vitro to identify potential targets of the tyrosine-specific protein kinase activity of pp60$^{src}$. It was anticipated that the targets of pp60$^{src}$ would be located at or near the membrane, since pp60$^{src}$ in fetal brain is associated exclusively with the particulate fraction (Maness et al., 1988). Moreover, the transforming ability of pp60$^{src}$, the closely related tyrosine kinase encoded by the Rous sarcoma virus src gene, depends on its membrane association (Fujii et al., 1989). Two major $^{32}$P-labeled membrane proteins of 55 and 53 kD were seen as charge trains near internal standards, pl 4.6–4.7 (Fig. 1 B). The 55- and 53-kD proteins partly overlapped with $\alpha$- and $\beta$-tubulin, localized on the same filter by immunostaining (Fig. 1 C). Phosphorylated $\alpha$-tubulin was slightly shifted to the acidic side due at least in part to charge differences, but perhaps also indicating phosphorylation of particular isoforms. Note that a different ampholine range was used in Fig. 1 A. When the 55- and 53-kD $^{32}$P-labeled proteins were analyzed individually for phosphoamino acid content, tyrosine was the principal residue phosphorylated (Fig. 2 A), and

**Figure 3.** Inhibition of endogenous tubulin phosphorylation in growth cone membranes by a monoclonal antibody specific for pp60$^{src}$. A-Fraction membranes (15 $\mu$g) were preincubated with mAb 327 IgG or nonimmune IgG at 0, 0.11, or 0.35 mg/ml in 0.02 M Tris HC1 (pH 7.4), 50 $\mu$M Na$_3$VO$_4$, 100 KIU/ml aprotinin, and 0.01% leupeptin (48 $\mu$g) for 1 h at room temperature before carrying out the endogenous phosphorylation reaction in a final volume of 96 $\mu$l. The reaction was terminated in 15 mM Na EDTA and centrifuged in an airfuge at 4°C for 20 min at 30 psi. The membrane pellets were analyzed by SDS-PAGE. $\alpha$- and $\beta$-tubulin bands were excised and counted in Aquasol for $^{32}$P to quantitate the degree of inhibition (see text). Minor phosphoproteins were quantitated from darker film exposures using the volume integration function of a computing densitometer (model 300A; Molecular Dynamics). Lane 1, no IgG; lane 2, mAb 327 IgG (0.11 mg/ml); lane 3, mAb 327 IgG (0.35 mg/ml); lane 4, nonimmune IgG (0.11 mg/ml); lane 5, nonimmune IgG (0.35 mg/ml).
each subunit was not determined, nor was the extent of phosphorylation prior to initiating the in vitro phosphorylation reaction. These stoichiometries do not necessarily reflect the maximal steady-state levels of phosphorylation, as they are determined in part by the rate of turnover of phosphate on the protein and the extent to which phosphorylation sites were previously occupied. The 60-kD phosphoprotein at the extreme basic end of the autoradiogram (Fig. 1 B) was immunoprecipitated from extracts with a monoclonal antibody (mAb 327) specific for pp60^{src}. Other phosphoproteins of 92-, 48-, and 43-kD were present at lower levels (Fig. 1 B), and additional phosphoproteins of >205 and 34 kD were observed upon prolonged autoradiographic exposure. Phosphoamino acid analysis of each of these unidentified proteins revealed only phosphotyrosine.

To investigate whether pp60^{src} was the tyrosine kinase responsible for phosphorylating tubulin in growth cone-enriched membranes, A-fraction membranes were incubated with pp60^{src}-specific mAb 327 (purified IgG), to inhibit its tyrosine kinase activity, before initiating the endogenous membrane phosphorylation reaction. mAb 327 binds to an NH_{2}-terminal region of pp60^{src}, which is located within the exon 3 domain (Potts et al., 1988; Kato et al., 1986; Parsons et al., 1986). The rationale for the use of mAb 327 as a possible inhibitor of pp60^{src} kinase activity was based on a preliminary finding that mAb 327 inhibited NGF-induced neurite extension when microinjected into fused rat PC12 pheochromocytoma cells (Kremer, N., J.S. Brugge, and S. Halegoua; personal communication). The epitope recognized by this antibody is located within a highly conserved region of pp60^{src} termed src homology 3 (SH3), which from mutational analyses appears to confer negative regulation on its kinase activity (Kato et al., 1986; Potts et al., 1988). In our assay, when mAb 327 was preincubated with A-fraction membranes, phosphorylation of α- and β-tubulin was inhibited in the endogenous phosphorylation reaction (Fig. 3). Phosphoamino acid analysis indicated that only tyrosine phosphorylation was inhibited. Individual tubulin
Figure 5. Partial proteolytic digests of tubulin from growth cone membranes and tubulin phosphorylated by pp60^src in vitro. (A) α-Tubulin (lanes 1 and 2) and β-tubulin (lanes 5 and 6) from growth cone membranes were excised from SDS gels and digested with 2 (lanes 1 and 5) or 10 ng (lanes 2 and 6) of S. aureus V8 protease. Peptides were separated on a second 12% SDS gel (Cleveland et al., 1977), transferred to an Immobilon filter, and immunoblotted with polyclonal phosphotyrosine antibodies and 125I-labeled protein A. Bovine brain α (lanes 3 and 4) and β-tubulin (lanes 7 and 8) were phosphorylated by pp60^src in immune complex kinase reactions with [γ32P]ATP, resolved on SDS gels, and digested with 2 (lanes 3 and 7) or 10 ng (lanes 4 and 8) of S. aureus V8 protease in a second 12% SDS gel. Sizes of peptides were calculated by linear regression analysis of retardation factor values from the migration of protein standards. (B) (Top) Proteins in growth cone membranes (150 μg) were separated on a 9-cm SDS gel at pH 8.8, transferred to Immobilon, and the filter incubated with PBS (−) or 40 μg/ml carboxypeptidase A in PBS (+) for 1 h at 37°C. The filter was immunoblotted with polyclonal phosphotyrosine antibodies and 125I-protein A, and exposed for autoradiography. α- and β-tubulin were not resolved in this gel system. (Bottom) α- and β-tubulin in growth cone membranes (50 μg) were 32P-labeled in a membrane phosphorylation reaction, resolved by SDS gel electrophoresis, transferred to Immobilon, and incubated without or with carboxypeptidase A as described above. Band intensities on autoradiograms were quantitated using the volume integration function of a computing densitometer (model 300A; Molecular Dynamics). Quantitation of band intensities corrected for exposure time and 32P decay revealed no decrease after carboxypeptidase treatment.
to occur as a postlysis consequence of endogenous tyrosine kinase activity. The phosphorylation of tubulin at tyrosine was estimated to be \( \approx 10\% \) from phosphotyrosine antibody immunoblotting, the amount of tubulin phosphorylated at tyrosine could be as much as 5\% of the total in the growth cone membrane fraction. Because \( \alpha \)- and \( \beta \)-tubulin subunits bind very tightly to each other (\( K_d < 1 \mu M; \) Detrich and Williams, 1978), tubulin dimers in RIPA were likely to be present during immunoprecipitation. Thus, the intensity of the autorigraph spots corresponding to tubulin subunits did not necessarily reflect the proportion of phosphorylated \( \alpha \)- and \( \beta \)-tubulin subunits. Moreover, it is possible that phosphotyrosine antibodies may not immunoprecipitate all forms of phosphorylated tubulin.

Membranes from the A-fraction exhibited a 36-fold enrichment in the phosphotyrosine-modified protein co-migrating with \( \alpha \)-tubulin, relative to its amount in homogenate membranes (Fig. 4 A, lanes 2 and 5). B- and C-fraction membranes, which contained smaller amounts of growth cone membrane, exhibited a lesser enrichment of phosphotyrosine-modified protein comigrating with \( \alpha \)-tubulin; 13- and 5-fold, respectively. The soluble fraction obtained from lysis of A-fraction particles contained no detectable phosphotyrosine-modified tubulin. Similar values were obtained when the samples were normalized to the amount of \( \alpha \)-tubulin in each fraction, relative to that in homogenate membranes, as determined by immunoblotting with excess tubulin monoclonal antibodies and \( ^{125}I \)-labeled Fab (23-, 8-, and 6-fold for the A-, B-, and C-fractions, respectively). This distribution approximated the 13-, 4-, and 3-fold enrichment of pp60\(^{src} \) in A-, B-, and C-fraction membranes relative to homogenate membranes (Maness et al., 1988). pp60\(^{src} \) in the B- and C-fractions includes that in lysed growth cone particles as well as some in neuritic shafts. Phosphotyrosine-containing protein comigrating with \( \beta \)-tubulin was also enriched in A-fraction membranes, but to a lesser degree than \( \alpha \)-tubulin. This phosphoprotein was also observed in homogenate membranes and the soluble fraction, in which phosphotyrosine-modified \( \alpha \)-tubulin was detectable only upon extended autoradiographic exposure. The molar ratio of protein A bound to phosphotyrosine antibodies and Fab IgG bound to tubulin antibodies on the filter was calculated from the \( ^{125}I \) cpm and specific radioactivities to estimate the relative levels of phosphorylation of \( \alpha \)- and \( \beta \)-tubulin (0.07 mol phosphate/mol \( \alpha \)-tubulin; 0.03 mol phosphate/mol \( \beta \)-tubulin). While subject to error due to differential affinity or accessibility of antibodies, the results indicate that the stoichiometry of tubulin phosphorylation in growth cone membranes in vivo approached the extent phosphorylated in membranes in vitro. Immunoblotting with excess tubulin monoclonal antibodies and \( ^{125}I \)-labeled Fab IgG was also used to estimate the total amount of tubulin in the subcellular fractions. This amount included both membrane and soluble tubulin. The A-, B-, and C-fraction membranes contained 3, 9, and 2\% of the tubulin protein applied to the sucrose gradient. The soluble fraction recovered from the gradient supernatant and lysates of the A-, B-, and C-fractions contained the majority of the tubulin protein (70\%) (the remainder was removed during the preparation of salt-washed membranes). Thus, phosphotyrosine-modified tubulin was limited to a minor subpopulation of the total tubulin in fetal rat brain.

The phosphorylation of tubulin at tyrosine did not appear to occur as a postlysis consequence of endogenous tyrosine kinase activity in A-fraction membranes. When fetal rat
brain extracts were lysed in the presence of N-ethylmaleimide (10 mM) or the nonhydrolyzable ATP analogue β, γ-methylene adenosine 5′-triphosphate (10 mM), both of which inhibit the activity of pp60−src (Maness, P. F., unpublished results), no decrease in the level of phosphotyrosine-modified tubulin was observed by immunoblotting. Addition of GTP (1 mM) to the homogenization buffer was similarly without effect despite its potential to attenuate denaturation of tubulin that might occur during the fractionation. Finally, inclusion of [γ-32P]ATP in the lysis buffer did not result in significant levels of incorporation of 32P into tubulin.

To gain information concerning the site(s) of phosphorylation, α- and β-tubulin from growth cone membranes were subjected to partial proteolysis with Staphylococcus aureus V8 protease and phosphopeptides analyzed by mapping by Cleveland et al. (1977) and immunoblotting with phosphotyrosine antibodies. These antibodies recognized an initial α-tubulin cleavage product of 38 kD (Fig. 5 A, lane 1). The 38-kD phosphopeptide was further digested to peptides of 28, 20, 14, and 13 kD, each of which contained phosphotyrosine epitopes. V8 protease digestion of β-tubulin from growth cone membranes in vivo revealed a similar initial cleavage peptide of ~37 kD that was recognized by phosphotyrosine antibodies (Fig. 5 A, lane 8). Further digestion produced a number of smaller fragments.

The carboxyl-terminal tyrosine residue in α-tubulin can be enzymatically removed and religated in vivo (Barra et al., 1974), and is known to be present in growth cones, where microtubule populations are dynamic (Wehland and Weber, 1987). This residue did not appear to be phosphorylated either in vivo or in vitro, as carboxypeptidase A treatment of α-tubulin from growth cone membranes did not reduce the amount of phosphotyrosine detectable with phosphotyrosine antibodies, nor did it reduce the amount of 32P in α-tubulin after membrane phosphorylation in vitro (Fig. 5 B). In both cases, the terminal tyrosine residues appeared to be completely removed by carboxypeptidase A treatment, as indicated from immunoblotting (not shown) with peptide antibody YL1/2, which is specific for the tyrosinated α-tubulin (Kilmartin et al., 1982). These results notwithstanding, the terminal tyrosine residue of α-tubulin could be a site for phosphorylation, if neither carboxypeptidase A nor YL1/2 antibody recognized the epitope containing phosphorylated tyrosine.

Tyrosine Phosphorylation of Tubulin in Primary Neuronal Cultures

To provide an independent means of demonstrating the presence of phosphotyrosine-modified tubulin in neuronal cells in vivo, phosphoproteins were labeled with [32P]orthophosphate in primary cultures of cortical neurons prepared from brains of fetal rats at the same gestational age at which growth cone membranes were isolated. These cultures contained 70–80% neuronal cells, as identified by immunofluorescence staining with neurofilament protein antibodies (Wood and Anderton, 1981). 32P-labeled proteins in whole cell lysates comigrated with α- and β-tubulin on two-dimensional gels optimized for resolving tubulin isoforms (Fig. 6 A). Alkaline treatment, which causes partial hydrolysis of phosphoserine residues (Cooper et al., 1983), revealed by this enrichment eight phosphoproteins in the α-tubulin region and two minor phosphoproteins in the β-tubulin region (Fig. 6 B). 32P-labeled α-tubulin isoforms appeared to be a minor subset of α-tubulin, indicated by their limited degree of overlap with α-tubulin immunostaining (Fig. 6 C). 32P-labeled β-tubulin was similarly restricted to a subpopulation of β-tubulin. Alkaline treatment reduced the 32P content of the most basic β-tubulin species, which is probably the isoform phosphorylated at serine in differentiating neuroblastoma cells (Gard and Kirschen, 1985). Other alkaline-resistant phosphoproteins in the cultures could be divided into four major groups: (a) an acidic cluster of 92-kD phosphoproteins, (b) an acidic cluster of 42-kD phosphoproteins, (c) a large cluster of phosphoproteins of 70–80 kD (pI 4.7–5.3), and (d) a trio of phosphoproteins of ~40 kD (pI 4.6–4.7). The phosphotyrosine content of these proteins was not determined.

The individual alkaline-resistant phosphoproteins in the tubulin region were not sufficiently labeled to support phosphoamino acid analysis, but when the entire regions demarcated by α- or β-tubulin immunostaining were analyzed for phosphoamino acid content, phosphotyrosine accounted for 35% of the 32P-labeled phosphoamino acids in α-tubulin; 24% in β tubulin. Phosphoserine and phosphothreonine accounted for 46 and 19% of the labeled phosphoamino acids in α-tubulin; 60 and 16% in β-tubulin. Tyrosine-phosphorylated α-tubulin was more abundant than β-tubulin in the neuronal cultures, as it was in A-fraction membranes analyzed by immunoblotting with phosphotyrosine antibodies (Fig. 4 A). In contrast, nearly equivalent amounts of α- and β-tubulin were phosphorylated at tyrosine in the endogenous phosphorylation reaction with A-fraction membranes in vitro. This difference may reflect the in vitro conditions of membrane phosphorylation, or alternatively, the presence of soluble factors that could modulate the activity or substrate specificity of pp60−src in vivo.

Direct Phosphorylation of Purified Tubulin at Tyrosine by pp60−src

To determine if pp60−src was capable of phosphorylating tubulin directly, pp60−src was immunoprecipitated with mAb GD11 from an extract of A-fraction membranes, and incubated with tubulin purified from adult bovine brain in an immune complex protein kinase assay (see Materials and Methods). mAb GD11 binds very close to the mAb327 site within the NH2-terminal region of pp60−src (Parsons et al., 1986). pp60−src phosphorylated tubulin subunits with a stoichiometry of 2.8 × 10−4 mol phosphotyrosine/mol α-tubulin and 4.5 × 10−4 mol phosphotyrosine/mol β-tubulin, under conditions at which maximal incorporation of 32P into each subunit was reached as determined from pilot experiments. Phosphoamino acid analysis revealed only phosphotyrosine in each subunit (Fig. 2 B). No phosphorylation of tubulin was observed by contaminating kinases in reactions without pp60−src, or when normal mouse IgG was substituted for mAb GD11. The relatively low stoichiometry of tubulin phosphorylation in immune complexes compared to membranes probably resulted from partial inhibition of pp60−src in the immune complex, rather than from a difference in tubulin isoforms in fetal rat and adult bovine brain to serve as substrates. This was shown by the observation that bovine brain tubulin was efficiently phosphorylated at tyrosyl residues when added to the endogenous phosphoryla-
Figure 6. \(^{32}\)P-labeling of tubulin in cultures of rat cortical neurons in vivo. \(^{32}\)P-labeled proteins in whole cell lysates from \(~3 \times 10^6\) cells in rat cortical neuron cultures were separated by two-dimensional gel electrophoresis optimized for resolution of tubulin isoforms (1.5% ampholines [pH 4–6], 0.5% ampholines [pH 3–10]) and transferred to an Immobilon filter. (A) Filter exposed 12 h for autoradiography reveals phosphorylated tubulin species. (B) The same filter treated with alkali to enrich for phosphotyrosine-modified protein, then exposed 8 d for autoradiography, reveals alkali-resistant forms of phosphorylated tubulin. (C) The same filter, before alkali treatment, was immunostained with \(\alpha\)- (DM-1A) and \(\beta\)-tubulin (TUB 2.1) monoclonal antibodies using horseradish peroxidase–conjugated secondary antibodies, and reveals the migration of phosphorylated and unphosphorylated tubulin species. The tubulin antibodies were used sequentially in preliminary studies to allow unambiguous identification of \(\alpha\)- and \(\beta\)-tubulin species. Small arrows in B indicate the position of alkali-resistant forms of \(^{32}\)P-labeled \(\alpha\)-tubulin; large arrowheads indicate those forms of \(\beta\)-tubulin. Arrows in A and C were placed to match exactly their positions in B. Internal standards were the same as in Fig. 1.

Partial V8 protease digestion of bovine brain \(\alpha\)-tubulin phosphorylated by pp60\(^{src}\) in the immune complex protein kinase reaction (Fig. 5 A, lanes 3 and 4) revealed a pattern of phosphopeptides (38, 28, 20, and 14 kD), similar to those

\[\text{reaction reaction with A-fraction membranes (0.058 mol phosphotyrosine/mol} \ \alpha\text{-tubulin; 0.054 mol phosphotyrosine/mol} \ \beta\text{-tubulin), approximating the stoichiometry of phosphorylation of fetal rat brain tubulin in A-fraction membranes.} \]
observed in vivo, with an additional peptide of 18 kD. V8 protease digestion of β-tubulin after phosphorylation by pp60src (Fig. 5 A, lanes 7 and 8) also produced major phosphopeptides of sizes similar to those phosphorylated in vivo, as well as an ~20-kD phosphopeptide. These results showed that pp60src from the growth cone membrane fraction was capable of directly phosphorylating α- and β-tubulin, and that similar phosphopeptides were phosphorylated in vivo and in vitro.

Discussion
An analysis of protein tyrosine kinase substrates in growth cone–enriched membranes from fetal rat brain has revealed a novel posttranslational modification of tubulin. Subpopulations of α- and β-tubulin were the major proteins phosphorylated at tyrosyl residues in growth cone membrane preparations in vitro and in vivo. Tubulin tyrosine phosphorylation appeared to be the consequence of the activity of the tyrosine kinase pp60src, which is enriched in the growth cone membrane fraction. These results represent the first demonstration of phosphorylated tyrosine residues in tubulin in vivo. The availability of a subcellular fraction enriched in membranes derived from growth cones and neuritic shafts, and containing only 0.2% of total protein in fetal brain homogenate, facilitated the detection of phosphotyrosine-modified tubulin, which constituted a minor proportion of tubulin protein in fetal rat brain. It is not known whether the forms of tubulin phosphorylated by pp60src in vivo represent particular isoforms encoded by members of the tubulin multigene family (Cleveland and Sullivan, 1985), or are posttranslationally modified forms. Phosphorylation of tubulin in vivo has been demonstrated previously only on serine residues of a basic β-tubulin isoform, during serum withdrawal-induced differentiation of N115 mouse neuroblastoma cells (Gard and Kirschner, 1985). Tubulin in these neuroblastoma cells may lack intrinsic phosphotyrosine, or else phosphotyrosine residues became dephosphorylated during cell lysis, because the cells were not treated with sodium orthovanadate, a reagent that greatly enhances the detection of phosphotyrosine-modified proteins by inhibiting the ordinarily rapid dephosphorylation of proteins by tyrosine phosphatases (Brown and Gordon, 1984; Collett et al., 1984; Yonemoto et al., 1987).

Tubulin is known to be an in vitro substrate of several protein kinases in brain, and in certain cases, phosphorylation inhibits polymerization. Casein kinase II, cAMP-dependent, and Ca2+/calmodulin-dependent protein kinases have been shown to phosphorylate tubulin on serine and threonine residues in vitro (Slodoba et al., 1975; Serrano et al., 1987; Goldenring et al., 1984; Katz et al., 1985). Phosphorylation by the Ca2+/calmodulin-dependent kinase was reported to block in vitro polymerization of tubulin (Wandosell et al., 1986). Tubulin is also phosphorylated at tyrosine in vitro by high amounts of the insulin receptor tyrosine kinase, inhibiting polymerization (Kadawaki et al., 1985; Wandosell et al., 1987), and by the epidermal growth factor receptor kinase (Chinkers and Cohen, 1981; Akiyama et al., 1986). Studies are in progress to determine whether phosphorylation of tubulin at tyrosine by pp60src from the nerve growth cone membrane preparation alters its ability to polymerize or to bind to microtubule-associated proteins.

An unresolved question is whether or not the viral src product phosphorylates tubulin in vivo. The viral src tyrosine kinase has also been shown to phosphorylate tubulin in vitro, however, with no evidence for in vivo phosphorylation in Rous sarcoma virus–transformed cells that had not been treated with orthovanadate (Levinson et al., 1980; Gilmer and Erikson, 1981; Maness and Levy, 1983). Clearly, pp60src can induce neurite extension, a process that depends on microtubule formation, as demonstrated by the ability of the viral src protein to induce neuronal differentiation in rat PC12 pheochromocytoma cells (Alemá et al., 1985).

Evidence presented here supports the conclusion that pp60src is the tyrosine kinase that phosphorylates tubulin in the nerve growth cone preparation. Membranes from the growth cone–enriched fraction contained a protein tyrosine kinase that phosphorylated membrane–associated tubulin in vitro relatively efficiently, and this phosphorylation was significantly reduced by monoclonal antibodies specific for pp60src. Furthermore, tubulin phosphorylation was not stimulated in growth cone membranes in vitro by the addition of ligands that are known to activate other receptor/tyrosine kinases. pp60src in immune complexes from growth cone membranes directly phosphorylated purified tubulin in vitro. The much lower stoichiometry of tubulin phosphorylation by pp60src in immune complexes compared to growth cone membranes may be attributed to the inhibitory effect of the src monoclonal antibody. This antibody may constrain kinase activity upon binding to the putative negative regulatory domain of pp60src (Kato et al., 1986; Potts et al., 1988).

The finding that phosphopeptides of tubulin phosphorylated by pp60src in vitro were similar to those observed in vivo lends further support to the interpretations that pp60src is the enzyme responsible for phosphorylating tubulin in growth cones. Results of tubulin phosphopeptide analysis indicated a limited number of sites for tyrosine phosphorylation. The data for α-tubulin suggest that the terminal tyrosine residue is not phosphorylated. β-Tubulin, which is >40% homologous to α-tubulin (Mandelkow and Mandelkow, 1989), produced a phosphopeptide pattern similar to α-tubulin, suggesting that analogous residue(s) may be subject to phosphorylation by pp60src.

The stoichiometry of phosphate incorporation into tubulin in endogenous phosphorylation reactions (0.068–0.045 mol/mol α-, β-tubulin) was in good agreement with that estimated in vivo (0.07–0.03 moles/mole). Although somewhat low, these levels could be functionally significant, especially if a subpopulation of tubulin isoforms were phosphorylated, as suggested by the incomplete overlap of phosphorylated tubulin species with total tubulin on two dimensional gels. A small amount of phosphorylated tubulin incorporated into the ends of growing oligomers or microtubules could for example, have major local effects on properties such as tubulin polymerization or association with membranes.

The partitioning of phosphotyrosine-modified tubulin to the membrane fraction, even after high salt and saponin treatment, suggests a tight association with growth cone membranes. This association was not examined in detail, and could be an artifact of subcellular fractionation. However, characterization of membrane-associated tubulin in other systems supports the existence of a unique membrane-associated tubulin population that may carry out analogous
functions in growth cones (reviewed by Stephens, 1986, 1990). For example, a transport mechanism was postulated whereby membrane-bound tubulin dimers attach an organelle or vesicle to microtubules via microtubule-associated proteins or force-transducing molecules such as dynein or kinesin (Stephens, 1986). In the motile growth cone, tyrosine phosphorylation of tubulin could conceivably modulate transport of vesicle components to or from the cell's leading edge.

The presence of phosphotyrosine-modified tubulin in growth cones suggests other links between tyrosine kinase action, tubulin phosphorylation, and regulation of nerve growth cone function that do not require the existence of membrane-bound tubulin. Although soluble phosphotyrosine-modified tubulin was not detected, it is possible that some amount may be present in the growth cone cytoplasm in vivo. There is ample evidence that tubulin is assembled into axonal microtubules at the growth cone (Bamburg et al., 1986; Mitchison and Kirschner, 1988), and that some microtubules can extend far forward to the leading growth cone margin (Forscher and Smith, 1988), terminating near microfilament bundles at the bases of filopodia or lamellipodia (Letourneau and Ressler, 1983). Tyrosine phosphorylation of tubulin at or near the leading edge by pp60src could conceivably alter its assembly characteristics locally to regulate microtubule dynamics at the growth cone.

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