# Tubulin Is Phosphorylated at Tyrosine by pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ in nerve growth cone membranes. Phosphotyrosine antibodies were used to demonstrate phosphotyrosyl residues in a subpopulation of $\alpha$- and $\beta$-tubulin that was highly enriched in a subcellular fraction of growth cone membranes from fetal rat brain. The presence of phosphotyrosine-modified isoforms of $\alpha$- and $\beta$-tubulin in vivo was confirmed by $^{32}P$ 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 $\alpha$-tubulin and 0.045 mol phosphotyrosine/mol $\beta$-tubulin), and phosphorylation was specifically inhibited by antibodies directed against pp60$^{c\text{-}src}$, which is localized in the growth cone membranes. pp60$^{c\text{-}src}$ 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 $\alpha$- and $\beta$-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 Kirscher, 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$^{c\text{-}src}$, 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$^{c\text{-}src}$ action in the growth cone is indicated from our recent finding that pp60$^{c\text{-}src}$ is highly enriched in nerve growth cone membrane preparations (Maness et al., 1988). This localization is consistent with the onset of pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ in neuronal cells is further underlined by the presence of a unique, more active form of pp60$^{c\text{-}src}$ (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$^{c\text{-}src}$ is the predominant species in growth cone membranes (Maness et al., 1988).

The identification of intracellular targets of pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ 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-*abl* protein expressed in bacteria and affinity purified on phosphotyrosine-Sepharose (Wang, 1985; Wang and Baltimore, 1985). Monoclonal antibodies specific for phosphotyrosine (PY20) (Glenney et al., 1988), $\alpha$-tubulin (DM-1A), and $\beta$-tubulin (DM-1B) were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Monoclonal antibodies specific for $\beta$-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$^{c\text{-}src}$ were mAb327 (Lipsich 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 Pfenninger 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 (Pfenninger 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 $Na_2SO_4$, 20 mM Hepes (pH 7.4) then in 20 mM Tris-HCl, 10 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM Na-EGTA, 1 μg/ml pepstatin A (pH 7.4); and resuspension in 20 mM Tris-HCl, 1 mM $MgCl_2$, 1 μg/ml pepstatin A (pH 7.4). Buffers were supplemented with 10 mM NaF and 200 μM $Na_3VO_4$ to inhibit tyrosine phosphatases, and aprotinin (100 KIU/ml) and 0.01% leupeptin to prevent proteolysis. Protein was measured by the method of Lowry et al. (1951).

## Endogenous Phosphorylation of Proteins in Growth Cone Membranes

Membranes from the growth cone-enriched A-fraction (30 μg) were incubated for 40 min at 30°C with 20 mM Tris-HCl, 40–200 μM Na-ATP, 7.5 μCi [γ-$^{32}$P]ATP (3,000 Ci/mmol), 5 mM $MnCl_2$, 5 mM dithiothreitol, 1 μM protein kinase A inhibitor peptide (Scott et al., 1985; from R. Lefkowitz [Duke University, Durham, NC], 50 μM $Na_3VO_4$, 100 KIU/ml aprotinin, 0.01% leupeptin (pH 7.4) in a final volume of 30 μl. Proteins were separated by two-dimensional gel electrophoresis, electrophoretically transferred to an Immobilon filter (Millipore Continental Water Systems, Bedford, MA) (Burnette, 1981), and subjected to autoradiography at −70°C using Cronex 6-Plus x-ray film (Dupont Photo Products, Burbank, CA) with intensifying screens. To measure stoichiometry of tubulin phosphorylation, α- and β-tubulin were excised from Immunobilon filters and counted for $^{32}$P by liquid scintillation in Omnifluor (New England Nuclear, Boston, MA). The amount of membrane-associated tubulin in 30 μg of A-fraction membranes was estimated to be 1.5 (in the range of 0.75–3.0) μg based on the intensity of silver staining in comparison to serially diluted rat brain tubulin standards.

## Gel Electrophoresis, Immunoblotting, and Immunoprecipitation

For one-dimensional gel electrophoresis, proteins were separated by SDS-PAGE at pH 9.2, under reducing conditions, on slab gels (20 cm) containing 10% polyacrylamide, and a 1/37 weight ratio of *bis*-acrylamide to acrylamide (Laemmli, 1970). SDS (lauryl sulfate; Sigma Chemical Co.) contained tetradecyl and hexadecyl sulfate, which produced optimal resolution of α- and β-tubulin subunits (Best et al., 1981). For two-dimensional electrophoresis (O'Farrell, 1975), proteins were solubilized at 37°C for 2 min in 9.5 M urea, 2% (wt/vol) 3-[(3-cholamidopropyl) dimethylammonio] 1-propanesulfonate, 0.3% SDS, 5% (vol/vol) β-mercaptoethanol containing either 2% (vol/vol) ampholines (pH 3–10) (Bio-Rad Laboratories, Richmond, CA), or a mixture of 1.5% (vol/vol) (pH 4–6) and 0.5% (vol/vol) ampholines (pH 3–10). Internal protein standards (Pharmacia, Uppsala, Sweden) were added, and IEF carried out in tube gels for 17 h at 400 V, followed by 2 h at 500 V. Second-dimension slab gels contained 8% polyacrylamide. Protein silver staining was achieved by the method of Oakley et al., (1980).

For phosphotyrosine immunoblots, proteins were transferred electrophoretically from polyacrylamide gels to Immobilon filters (Millipore Continental Water Systems). Filters were incubated for 1 h with 1% fish gelatin in 50 mM Tris-HCl, 150 mM NaCl, 0.5% (vol/vol) Tween-20 (pH 7.4), then for 2 h with polyclonal phosphotyrosine antibodies (0.5 μg/ml) in the same buffer. After washing in buffer without gelatin, filters were incubated for 1 h with 1 μCi/ml $^{125}$I-protein A (30 μCi/μg; Amersham Corp., Arlington Heights, IL), washed, dried, and exposed for autoradiography. Band intensities on x-ray film were quantitated using the volume integration function of a computing densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA). Film exposure was adjusted so that band intensities were within the linear range of response of the film to $^{125}$I (Maness et al., 1983). To demonstrate specificity, phosphotyrosine antibodies were preincubated in 40 mM *o*-phospho-L-tyrosine for 1 h at room temperature, then applied to filters in buffer containing 40 mM *o*-phospho-L-tyrosine.

For tubulin immunoblots, filters were incubated for 1 h with 1% BSA (Fraction V; Boehringer Mannheim Biochemicals Inc., Indianapolis, IN) in 10 mM Tris-HCl, 150 mM NaCl, 0.05% (vol/vol) Tween-20 (pH 7.2), then for 2 h with a 1:250 dilution of antibody DM-1A and a 1:50 dilution of antibody TUB 2.1 in the same buffer. After washing, filters were incubated for 1 h with 1 μCi/ml $^{125}$I-sheep anti-mouse F(ab)'$_2$ fragment (5–20 μCi/μg; Amersham Corp.). $^{125}$I-labeled tubulin was quantitated by counting excised pieces of filter for gamma radiation. For experiments using alkaline phosphatase-conjugated secondary antibodies, Immobilon filters were incubated for 2 h with a 1:1,000 dilution of sheep anti-mouse IgG-alkaline phosphatase (Sigma Chemical Co.). Reaction product was developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt according to the Bio-Rad Laboratories manual. For experiments using horseradish peroxidase-conjugated secondary antibodies, filters were incubated for 1 h with 0.47 μg/ml biotinylated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Inc.) in 1% BSA, 10 mM Tris-HCl, 150 mM NaCl, 0.05% (vol/vol) Tween-20 (pH 7.2), washed, and incubated with streptavidin-horseradish peroxidase (0.42 μg/ml) (Bethesda Research Laboratories, Gaithersburg, MD) for 30 min in 1% BSA, 50 mM Tris-HCl, 150 mM NaCl (pH 7.4). Filters were washed in the same buffer without BSA, and color was developed at the site of immune complexes by incubating with 0.5 mg/ml 3,3'-diaminobenzidine HCl dihydrate and 0.02% (vol/vol) $H_2O_2$.

pp60$^{c\text{-}src}$ was detected on filters with mouse mAb 327 and $^{125}$I-sheep anti-mouse F(ab)'$_2$ fragment as described by Matten and Maness (1987).

Tubulin was immunoprecipitated from an extract of A-fraction membranes (50 μg) in RIPA (1% Na-deoxycholate, 1% (wt/vol) NP-40, 0.1% (wt/vol) SDS, 5 mM Na-EDTA, 1 mM Na-EGTA, 200 μM $Na_3VO_4$, 10 mM NaF, 100 KIU/ml aprotonin, 0.1% leupeptin, 10 mM Tris-HCl [pH 7.2]) using monoclonal antibody PY20 (6 μg) or normal mouse IgG (6 μg) and protein A–Sepharose beads as described in Matten and Maness (1987). Proteins were eluted from the beads with 40 mM phenyl phosphate in RIPA.

## Preparation and $^{32}$P Labeling of Primary Neuronal Cultures

Brains were dissected from 18-d fetal rats, and cells were dispersed for culture by a modification of the procedure of Banker and Cowan (1977). Cells were plated on 60-mm tissue culture dishes coated with poly-D-lysine, and incubated in MEM, 0.45% glucose, 5% FCS, 5% heat-inactivated horse serum, 5 U/ml penicillin, and 5 μg/ml streptomycin. The next day, cells were rinsed with $Ca^{2+}$, $Mg^{2+}$-free Hank's balanced salt solution containing 0.5% glucose, 5 U/ml penicillin, 5 μg/ml streptomycin, and grown at 37°C in 5% $CO_2$ for 3 d in N2 medium (Bottenstein and Sato, 1979). Cells were then radiolabeled for 12 h in 2 ml phosphate-free MEM, 2% nondialyzed FCS, 0.45% glucose, 20 μg/ml gentamycin, 2 mCi [$^{32}$P]orthophosphate (HCl free; 285 Ci/mmol; ICN Biomedicals, Inc.). During the final 2 h of incubation 100 μM $Na_3VO_4$ was added. Extracts were prepared for two-dimensional gel electrophoresis after rinsing in Hank's balanced salt solution.

## Phosphorylation of Purified Tubulin by pp60$^{c\text{-}src}$

Tubulin was purified from bovine brain by two cycles of polymerization/depolymerization and chromatography on phosphocellulose, then stored at −70°C (Weingarten et al., 1975; Zeeberg et al., 1980). Tubulin appeared free of contamination with microtubule-associated proteins by silver staining after SDS-PAGE. After thawing, polymerization-competent tubulin was selected by an additional cycle of polymerization/depolymerization. Tubulin concentration was quantitated spectrophotometrically from absorbance at 278 nm using an extinction coefficient of 1.2 A/(mg/ml) (Jacobs et al., 1974). A 1:1 mass ratio of α- and β-subunits was assumed. pp60$^{c\text{-}src}$ was immunoprecipitated with mAb GD11 and protein A–Sepharose from a detergent extract (250 μg) of A-fraction membranes (Matten and Maness,

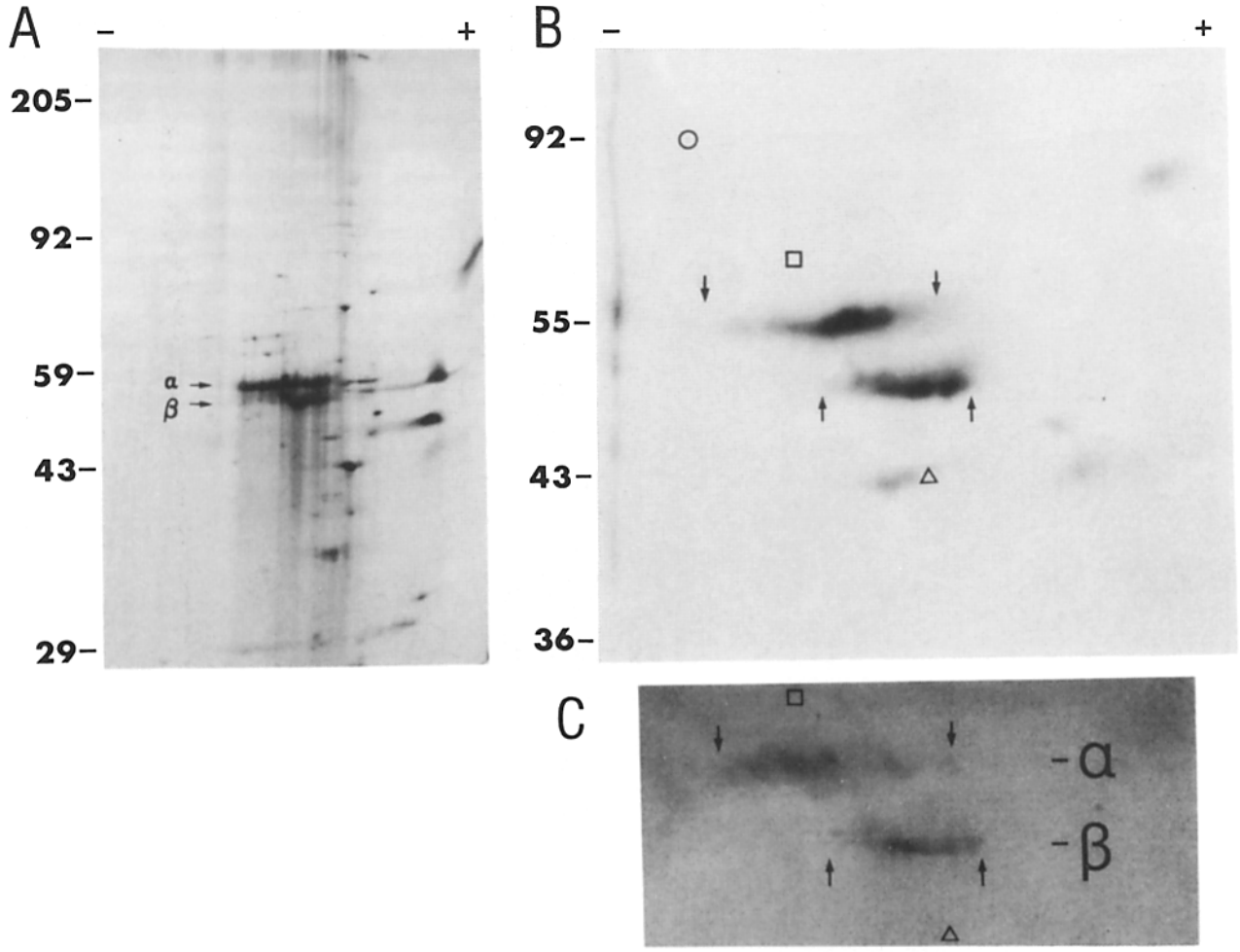


*Figure 1.* Endogenous phosphorylation of tubulin in growth cone–enriched membranes. (*A*) Silver-stained proteins in A-fraction membranes (30 μg) separated by two-dimensional gel electrophoresis; 2% ampholines (pH 3–10). (*B*) Proteins phosphorylated by endogenous phosphorylation in A-fraction membranes (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 (○) phosphorylase b (pI 5.6–5.8); (□) BSA (pI 4.7); (△) 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 [$\gamma$-$^{32}$P] ATP (17 Ci/mmol), 5 mM $MnCl_2$, 1 μM protein kinase A inhibitor peptide, 50 μM $Na_3VO_4$, 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 pp60$^{c\text{-}src}$ 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 $^{32}$P 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 Sefton, 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 $Na_2SO_4$ or saponin (Simkowitz et

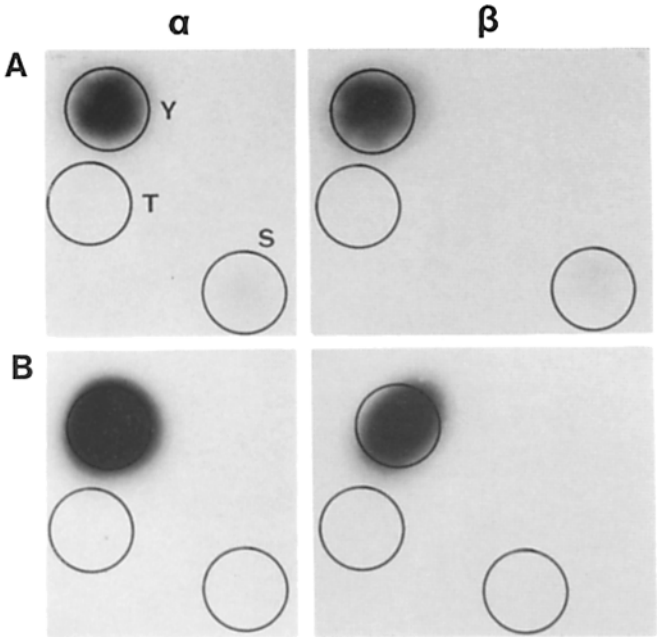


*Figure 2.* Phosphoamino acid analysis of tubulin phosphorylated in growth cone membranes or purified tubulin phosphorylated by pp60$^{c\text{-}src}$. (*A*) α- and β-tubulin in growth cone membranes (15 μg) were $^{32}$P labeled in a membrane phosphorylation reaction, resolved by SDS gel electrophoresis, transferred to Immobilon, and analyzed for phosphoamino acid content. (*B*) Bovine brain tubulin (8 μg) was phosphorylated by pp60$^{c\text{-}src}$ in the immune complex kinase assay, and α- and β-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 α- and β-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 [γ$^{32}$P]ATP in an endogenous protein kinase assay in vitro to identify potential targets of the tyrosine-specific protein kinase activity of pp60$^{c\text{-}src}$. It was anticipated that the targets of pp60$^{c\text{-}src}$ would be located at or near the membrane, since pp60$^{c\text{-}src}$ in fetal brain is associated exclusively with the particulate fraction (Maness et al., 1988). Moreover, the transforming ability of pp60$^{v\text{-}src}$, 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, pI 4.6–4.7 (Fig. 1 *B*). The 55- and 53-kD proteins partly overlapped with α- and β-tubulin, localized on the same filter by immunostaining (Fig. 1 *C*). Phosphorylated α-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 accounted for 82% of the $^{32}$P-labeled phosphoamino acids in the protein colocalizing with α-tubulin, and 80% of the phosphoamino acids in the protein colocalizing with β-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 α- and β-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 μM ATP, 30°C, 40 min). The phosphorylation of tubulin at tyrosyl residues, as determined from $^{32}$P incorporation, was 0.068 mol phosphotyrosine/mol α-tubulin and 0.045 mol phosphotyrosine/mol β-tubulin. The number of tyrosyl residues phosphorylated in

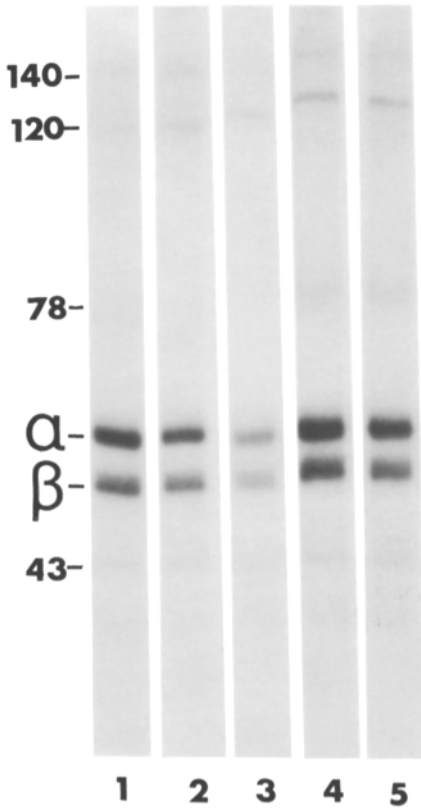


*Figure 3.* Inhibition of endogenous tubulin phosphorylation in growth cone membranes by a monoclonal antibody specific for pp60$^{c\text{-}src}$. A-Fraction membranes (15 μg) were preincubated with mAb 327 IgG or nonimmune IgG at 0, 0.11, or 0.35 mg/ml in 0.02 M Tris HCl (pH 7.4), 50 μM $Na_3VO_4$, 100 KIU/ml aprotinin, and 0.01% leupeptin (48 μl) for 1 h at room temperature before carrying out the endogenous phosphorylation reaction in a final volume of 96 μl. 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. α- and β-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).

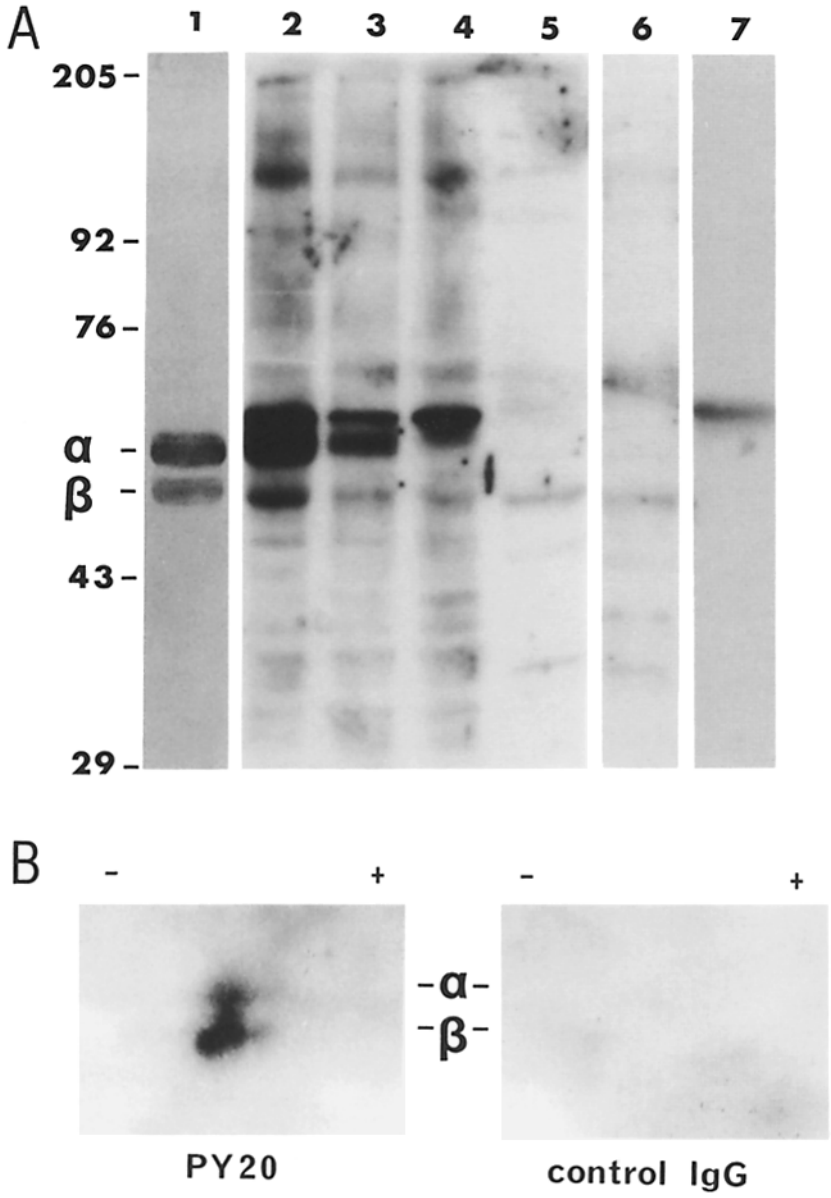


*Figure 4.* Proteins in growth cone–enriched membranes detected by immunoblotting with phosphotyrosine antibodies, and immunoprecipitation of tubulin with phosphotyrosine antibodies. (*A*) Protein (30 μg) in salt-washed membranes from the A-fraction (lanes *1*, *2*, and 7), B-fraction (lane *3*), C-fraction (lane *4*), homogenate (lane *5*), and the soluble fraction (lane *6*) were immunostained with α- (DM-1A) and β-tubulin (TUB 2.1) antibodies and alkaline phosphatase–conjugated secondary antibodies (lane *1*), or with polyclonal phosphotyrosine antibodies (lanes *2–6*) and $^{125}$I-protein A, or *src* mAb 327 and $^{125}$I-labeled secondary antibody (lane *7*). Autoradiography was for 11 d. (*B*) Proteins were immunoprecipitated from a RIPA extract of A-fraction membranes (50 μg) with monoclonal antibody PY20 or nonimmune mouse (control) IgG and protein A–Sepharose. Immunoprecipitated proteins were separated by two-dimensional electrophoresis under nonreducing conditions using 2% ampholines (pH 3–10), transferred to an Immobilon filter, and immunoblotted with monoclonal antibodies specific for α- (DM-1A) and β-tubulin (TUB 2.1) followed by $^{125}$I-labeled secondary antibody. Autoradiography was for 4 d. The positions of α- and β-tubulin from rat brain, which was included in the second dimension gel, are indicated.

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$^{c\text{-}src}$. 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$^{c\text{-}src}$ was the tyrosine kinase responsible for phosphorylating tubulin in growth cone–enriched membranes, A-fraction membranes were incubated with pp60$^{c\text{-}src}$-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$^{c\text{-}src}$, 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$^{c\text{-}src}$ 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$^{c\text{-}src}$ 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

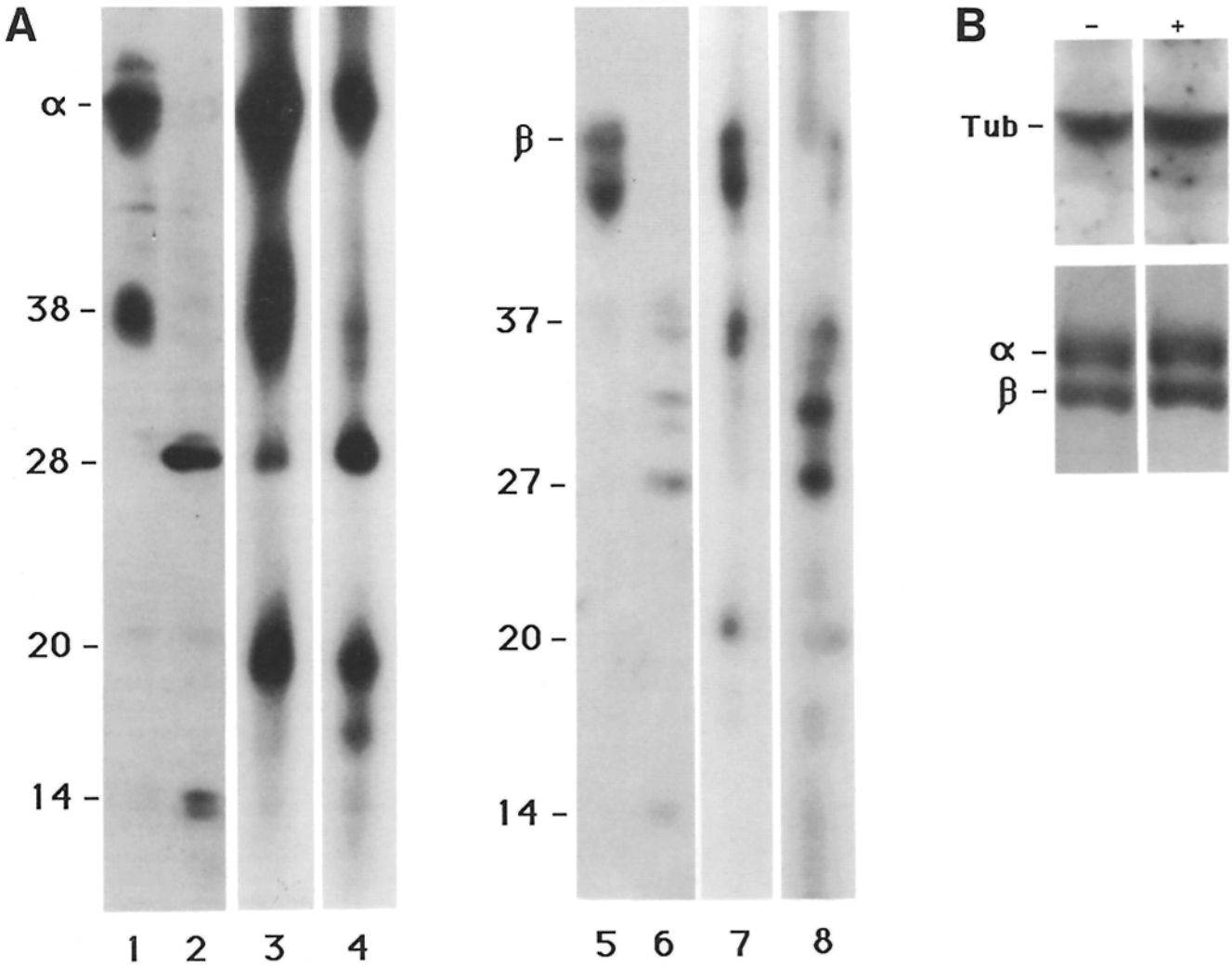


*Figure 5.* Partial proteolytic digests of tubulin from growth cone membranes and tubulin phosphorylated by pp60$^{c\text{-}src}$ in vitro. (*A*) α-Tubulin (lanes *1* and *2*) and β-tubulin (lanes *5* and *6*) from growth cone membranes were excised from SDS gels and digested with 2 (lanes *1* and *5*) 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 $^{125}$I-labeled protein A. Bovine brain α- (lanes *3* and *4*) and β-tubulin (lanes *7* and *8*) were phosphorylated by pp60$^{c\text{-}src}$ in immune complex kinase reactions with [γ$^{32}$P]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 $^{125}$I-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 (30 μg) were $^{32}$P-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 $^{32}$P decay revealed no decrease after carboxypeptidase treatment.

bands were excised from the gel and counted for $^{32}$P by liquid scintillation to determine the extent of inhibition. Tyrosine-specific phosphorylation was maximally inhibited in α- (74%) and β-tubulin (75%) at 0.35 mg/ml IgG with respect to the extent of tyrosine phosphorylation in untreated growth cone membranes. Less inhibition was observed in α- (45%) and β-tubulin (35%) at 0.11 mg/ml IgG. Nonimmune IgG had no effect up to 0.35 mg/ml, beyond which tubulin phosphorylation at serine, threonine, and tyrosine residues was partially inhibited. Even at 8 mg/ml mAb 327 did not completely inhibit tubulin tyrosine phosphorylation. The inability of mAb 327 to completely inhibit pp60$^{c\text{-}src}$ kinase activity is consistent with the ability of pp60$^{c\text{-}src}$ to phosphorylate exogenous substrates in mAb 327 immune complexes. A mouse monoclonal antibody (IgG) directed against S100 protein (Van Eldik and Zimmer, 1988) also produced no inhibition of phosphorylation of tubulin or other minor proteins (0.11–0.35 mg/ml). mAb 327 (0.35 mg/ml) also inhibited phosphorylation of 140-, 78-, and 43-kD proteins (84, 100, and 87%, respectively), suggesting that they,

too, were substrates of pp60$^{c\text{-}src}$. The 78-kD protein was more prominent in darker autoradiograms. The phosphorylation of the 120-kD protein was inhibited to a lesser extent (58%). These results provide evidence that pp60$^{c\text{-}src}$ is the kinase responsible for phosphorylating tubulin, either directly or indirectly, in growth cone–enriched membranes.

Other tyrosine-specific protein kinases were not detected in A-fraction membranes. Addition of insulin-like growth factor-I, insulin, epidermal growth factor, bombesin, or basic fibroblast growth factor (1–100 nM) to the endogenous membrane phosphorylation reaction did not stimulate tyrosine phosphorylation of tubulin, indicating that the tyrosine kinase receptors activated by these ligands probably did not contribute to tubulin phosphorylation. Moreover, there was no evidence for autophosphorylation of these receptors in growth cone membranes, as phosphorylation of high molecular mass proteins was not stimulated by addition of ligands to the membranes.

### *Identification of Phosphotyrosine-modified Tubulin in Growth Cone Membranes In Vivo*

To determine if tubulin was phosphorylated at tyrosine in vivo, membrane proteins from the growth cone–enriched A-fraction, which had not been subjected to in vitro phosphorylation, were subjected to immunoblotting with polyclonal phosphotyrosine antibodies (Wang, 1985). The most prominent phosphotyrosine-containing protein co-migrated with α-tubulin (55 kD) identified by immunostaining with α-tubulin antibodies (Fig. 4 *A*, lane *2*). Another major phosphotyrosine-modified protein (53 kD) overlapped the lower region of β-tubulin immunostaining. A third phosphotyrosine-modified protein was located above the α-tubulin immunostaining. Although this protein overlapped with pp60$^{c\text{-}src}$ (Fig. 4 *A*, lane *7*), its high level in the C-fraction (lane *4*), where pp60$^{c\text{-}src}$ is present at low levels, suggested that the band contained other phosphotyrosine-modified proteins. The 55- and 53-kD proteins probably did not represent multiple proteins, because they co-migrated with α- and β-tubulin in nonreducing electrophoresis conditions that altered the migration of α- and β-tubulin (not shown). Other phosphotyrosine-modified proteins, including those of 200, 150, 115, 92, 68, 45, and 37 kD, were observed at lower levels. Preincubation of the antibody with *o*-phospho-L-tyrosine blocked binding to all of the phosphotyrosine-containing proteins in A-fraction membranes, demonstrating that antibody recognition was specific (not shown). Phosphoserine or phosphothreonine did not block immunoreactivity, nor did acid treatment under conditions that hydrolyze phosphohistidine (Hultguist et al., 1966).

To confirm that the 55- and 53-kD phosphotyrosine-modified proteins were tubulin subunits, proteins from RIPA extracts of A-fraction membranes were immunoprecipitated with a monoclonal phosphotyrosine antibody (PY20), and analyzed by two-dimensional gel electrophoresis and immunoblotting with tubulin antibodies (Fig. 4 *B*). Proteins with molecular masses and isoelectric focusing properties characteristic of α- and β-tubulin were recognized by the tubulin antibodies and were specifically eluted with the phosphotyrosine analogue phenyl phosphate, demonstrating that tubulin in growth cone–enriched membranes contained phosphorylated tyrosyl residues. Considering the efficiency of immunoprecipitation of phosphorylated tubulin, which was estimated to be ~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 α- and β-tubulin subunits bind very tightly to each other ($K_d < 1$ μM; Detrich and Williams, 1978), tubulin dimers in RIPA were likely to be present during immunoprecipitation. Thus, the intensity of the autoradiographic spots corresponding to tubulin subunits did not necessarily reflect the proportion of phosphorylated α- and β-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 α-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 α-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 α-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$^{c\text{-}src}$ in A-, B-, and C-fraction membranes relative to homogenate membranes (Maness et al., 1988). pp60$^{c\text{-}src}$ 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 β-tubulin was also enriched in A-fraction membranes, but to a lesser degree than α-tubulin. This phosphoprotein was also observed in homogenate membranes and the soluble fraction, in which phosphotyrosine-modified α-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 α- and β-tubulin (0.07 mol phosphate/mol α-tubulin; 0.03 mol phosphate/mol β-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

brains were lysed in the presence of *N*-ethylmaleimide (10 mM) or the nonhydrolyzable ATP analogue $\beta$, $\gamma$-methylene adenosine 5'-triphosphate (10 mM), both of which inhibit the activity of pp60$^{c\text{-}src}$ (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 homogenation buffer was similarly without effect despite its potential to attenuate denaturation of tubulin that might occur during the fractionation. Finally, inclusion of [$\gamma^{32}$P]ATP in the lysis buffer did not result in significant levels of incorporation of $^{32}$P into tubulin.

To gain information concerning the site(s) of phosphorylation, $\alpha$- and $\beta$-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 $\alpha$-tubulin cleavage product of 38 kD (Figure 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 $\beta$-tubulin from growth cone membranes in vivo revealed a similar initial cleavage peptide of $\sim$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 $\alpha$-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 $\alpha$-tubulin from growth cone membranes did not reduce the amount of phosphotyrosine detectable with phosphotyrosine antibodies, nor did it reduce the amount of $^{32}$P in $\alpha$-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 $\alpha$-tubulin (Kilmartin et al., 1982). These results notwithstanding, the terminal tyrosine residue of $\alpha$-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 [$^{32}$P]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). $^{32}$P-labeled proteins in whole cell lysates comigrated with $\alpha$- and $\beta$-tubulin on two-dimensional gels optimized for resolving tubulin isoforms (Fig. 6 *A*). Alkali treatment, which causes partial hydrolysis of phosphoserine residues (Cooper et al., 1983), revealed by this enrichment eight phosphoproteins in the $\alpha$-tubulin region and two minor phosphoproteins in the $\beta$-tubulin region (Fig. 6 *B*). $^{32}$P-labeled $\alpha$-tubulin isoforms appeared to be a minor subset of $\alpha$-tubulin, indicated by their limited degree of overlap with $\alpha$-tubulin immunostaining (Fig. 6 *C*). $^{32}$P-labeled $\beta$-tubulin was similarly restricted to a subpopulation of $\beta$-tubulin. Alkali treatment reduced the $^{32}$P content of the most basic $\beta$-tubulin species, which is probably the isoform phosphorylated at serine in differentiating neuroblastoma cells (Gard and Kirschner, 1985). Other alkali-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 $\sim$40 kD (pI 4.6–4.7). The phosphotyrosine content of these proteins was not determined.

The individual alkali-resistant phosphoproteins in the tubulin region were not sufficiently labeled to support phosphoamino acid analysis, but when the entire regions demarcated by $\alpha$- or $\beta$-tubulin immunostaining were analyzed for phosphoamino acid content, phosphotyrosine accounted for 35% of the $^{32}$P-labeled phosphoamino acids in $\alpha$-tubulin; 24% in $\beta$ tubulin. Phosphoserine and phosphothreonine accounted for 46 and 19% of the labeled phosphoamino acids in $\alpha$-tubulin; 60 and 16% in $\beta$-tubulin. Tyrosine-phosphorylated $\alpha$-tubulin was more abundant than $\beta$-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 $\alpha$- and $\beta$-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$^{c\text{-}src}$ in vivo.

### *Direct Phosphorylation of Purified Tubulin at Tyrosine by pp60$^{c\text{-}src}$*

To determine if pp60$^{c\text{-}src}$ was capable of phosphorylating tubulin directly, pp60$^{c\text{-}src}$ 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 $NH_2$-terminal region of pp60$^{c\text{-}src}$ (Parsons et al., 1986). pp60$^{c\text{-}src}$ phosphorylated tubulin subunits with a stoichiometry of $2.8 \times 10^{-4}$ mol phosphotyrosine/mol $\alpha$-tubulin and $4.5 \times 10^{-4}$ mol phosphotyrosine/mol $\beta$-tubulin, under conditions at which maximal incorporation of $^{32}$P 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$^{c\text{-}src}$, 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$^{c\text{-}src}$ 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-

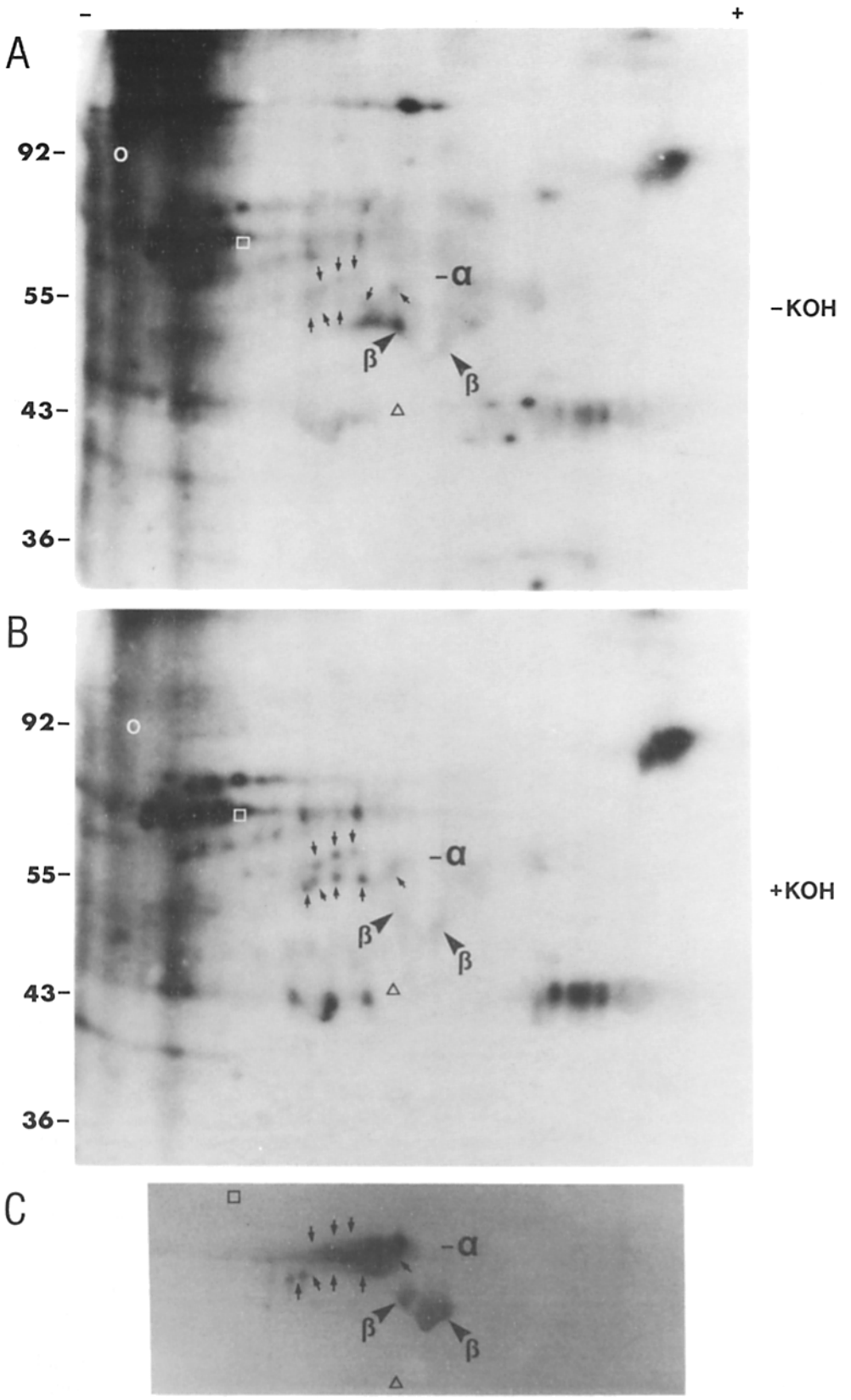


*Figure 6.* $^{32}$P-labeling of tubulin in cultures of rat cortical neurons in vivo. $^{32}$P-labeled proteins in whole cell lysates from ~3 × $10^5$ cells in rat cortical neuron cultures were separated by two-dimensional gel electrophoresis optimized for resolution of tubulin isoforms (1.5% ampholines [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 α- (DM-1A) and β-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 α- and β-tubulin species. Small arrows in *B* indicate the position of alkali-resistant forms of $^{32}$P-labeled α-tubulin; large arrowheads indicate those forms of β-tubulin. Arrows in *A* and *C* were placed to match exactly their positions in *B*. Internal standards were the same as in Fig. 1.

tion reaction with A-fraction membranes (0.058 mol phosphotyrosine/mol α-tubulin; 0.054 mol phosphotyrosine/mol β-tubulin), approximating the stoichiometry of phosphorylation of fetal rat brain tubulin in A-fraction membranes.

Partial V8 protease digestion of bovine brain α-tubulin phosphorylated by pp60$^{c\text{-}src}$ 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

observed in vivo, with an additional peptide of 18 kD. V8 protease digestion of β-tubulin after phosphorylation by pp60$^{c\text{-}src}$ (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 pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$, 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 pp60$^{c\text{-}src}$ 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 $Ca^{2+}$/calmodulin-dependent protein kinases have been shown to phosphorylate tubulin on serine and threonine residues in vitro (Sloboda et al., 1975; Serrano et al., 1987; Goldenring et al., 1984; Katz et al., 1985). Phosphorylation by the $Ca^{2+}$/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 pp60$^{c\text{-}src}$ 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, pp60$^{v\text{-}src}$ 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 pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$. 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. pp60$^{c\text{-}src}$ in immune complexes from growth cone membranes directly phosphorylated purified tubulin in vitro. The much lower stoichiometry of tubulin phosphorylation by pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$ (Kato et al., 1986; Potts et al., 1988).

The finding that phosphopeptides of tubulin phosphorylated by pp60$^{c\text{-}src}$ in vitro were similar to those observed in vivo lends further support to the interpretations that pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$.

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 $pp60^{c\text{-}src}$ could conceivably alter its assembly characteristics locally to regulate microtubule dynamics at the growth cone.

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