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Complexes of γ-tubulin with nonreceptor protein tyrosine kinases Src and Fyn in differentiating P19 embryonal carcinoma cells

Vitaliy Kukharskyy, Vadym Sulimenko, Libor Macůrek, Tetyana Sulimenko, Eduarda Dráberová, and Pavel Dráber*

Institute of Molecular Genetics, Department of Biology of Cytoskeleton, Academy of Sciences of the Czech Republic, Prague 4, Czech Republic

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Abstract

Nonreceptor protein tyrosine kinases of the Src family have been shown to play an important role in signal transduction as well as in regulation of microtubule protein interactions. Here we show that γ-tubulin (γ-Tb) in P19 embryonal carcinoma cells undergoing neuronal differentiation is phosphorylated and forms complexes with protein tyrosine kinases of the Src family, Src and Fyn. Elevated expression of both kinases during differentiation corresponded with increased level of proteins phosphorylated on tyrosine. Immunoprecipitation experiments with antibodies against Src, Fyn, γ-tubulin, and with anti-phosphotyrosine antibody revealed that γ-tubulin appeared in complexes with these kinases. In vitro kinase assays showed tyrosine phosphorylation of proteins in γ-tubulin complexes isolated from differentiated cells. Pretreatment of cells with Src family selective tyrosine kinase inhibitor PP2 reduced the amount of phosphorylated γ-tubulin in the complexes. Binding experiments with recombinant SH2 and SH3 domains of Src and Fyn kinases revealed that protein complexes containing γ-tubulin bound to SH2 domains and that these interactions were of SH2-phosphotyrosine type. The combined data suggest that Src family kinases might have an important role in the regulation of γ-tubulin interaction with tubulin dimers or other proteins during neurogenesis.
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Keywords: Antibodies; Fyn kinase; Gamma-tubulin; Neuronal differentiation; P19 cells; Src kinase

Introduction

It is well established that the development of nervous system is regulated by a variety of protein tyrosine kinases. While the neural functions of receptor tyrosine kinases are sufficiently characterized, the functions of nonreceptor tyrosine kinases, including kinases $pp60^{src}$ (Src) and $p59^{fyn}$ (Fyn) that belong to the family of Src kinases, are not fully understood. Src family kinases have been implicated in events regulating neuronal differentiation and function of neuronal cells [1–3]. The kinases could modulate microtubule dynamics during neurite outgrowth as a fraction of tubulin associated with plasma membrane serves as a substrate for Src kinase [4,5]. Both Src and Fyn kinases were found in complexes containing cell surface receptors and tubulin [6].

Embryonal carcinoma cell line P19 is a suitable model system for studying the molecular mechanisms underlying differentiation and early embryonic development. P19s are murine multipotent cells that can differentiate in culture into neural cells when aggregated and subsequently cultured in the presence of a nontoxic concentration of all-*trans*-retinoic acid (RA) [7]. There are substantial changes in the expression of microtubule proteins during neuronal differentiation of P19 cells [8–10], and both Src and Fyn kinases are present in differentiated cells [11].

Abbreviations: EC, embryonal carcinoma; Fyn, protein tyrosine kinase $p59^{fyn}$; GST, glutathione *S*-transferase; MTOC, microtubule organizing center; MSB, microtubule-stabilizing buffer; PBS, phosphate buffered saline; PP2, Src-family selective tyrosine kinase inhibitor; PP3, negative control for inhibitor PP2; P-Tyr, protein phosphorylated on tyrosine; RA, all-*trans*-retinoic acid; SDS-PAGE, one-dimensional SDS-polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional electrophoresis; Src, protein tyrosine kinase $pp60^{src}$; SH2 and SH3, Src homology 2 and 3 domains; α-Tb, α-tubulin; Acα-Tb, acetylated α-tubulin; βIII-Tb, β-tubulin class III; γ-Tb, γ-tubulin; γTuRC, γ-tubulin-ring complex; γTuSC, γ-tubulin small complex; TBST, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20.

* Corresponding author. Institute of Molecular Genetics, Department of Biology of Cytoskeleton, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Fax: +420-241-062-758.
E-mail address: paveldra@biomed.cas.cz (P. Dráber).

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Microtubules, known to play an essential role during neurite outgrowth, are dynamic polymers assembled from α- and β-tubulin heterodimers. γ-Tubulin (γ-Tb) [12] is a highly conserved member of the tubulin superfamily that is located on the minus end of microtubules in microtubule organizing centers (MTOCs) [13]. The majority of γ-tubulin is, however, associated with other proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complexes (γTuRCs) were identified in various species [14,15]. Besides γTuRC, there also exist smaller complexes (γ-tubulin small complex; γTuSC) [16] that comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins) [17,18]. The γ-TuRCs are formed by small complexes and by other proteins. In addition to nucleation from MTOC, γ-TuRCs are also involved in the regulation of the dynamics of microtubule minus ends [19].

γ-Tubulin itself has been recognized as a microtubule minus end binding molecule in not anchored microtubules [20], and the binding sites for α- and β-tubulin subunits on γ-tubulin were identified using synthetic peptides [21]. In brain, soluble γ-tubulin associated with αβ-tubulin dimers irrespective of the size of γ-tubulin complexes [22]. Membrane-bound forms of γ-tubulin with nucleation activity were also described [23,24].

While it is well known that the αβ-tubulin heterodimers are subject to a large number of posttranslational modifications that are essential for regulation of their functions [25], knowledge about posttranslational modifications of γ-tubulin and its interaction partners is limited. Phosphorylation of γ-tubulin on tyrosine has so far been reported in budding yeast [26], and we found γ-tubulin in rat basophilic leukemia cells in complexes containing protein tyrosine kinase p53/p56^{lyn} belonging to the family of Src kinases [27]. We therefore wanted to find out whether γ-tubulin also interacts with other Src family kinases that are expressed during neuronal differentiation. During this process, substantial changes in cell morphology and microtubule arrangement are known to occur.

Here we report for the first time that γ-tubulin in differentiating P19 cells is phosphorylated and forms complexes with nonreceptor protein tyrosine kinases Src and Fyn. Consequently, the Src family kinases might have an important role in the regulation of γ-tubulin interaction with tubulin dimers or other proteins during neuronal differentiation.

Materials and methods

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus, and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL, USA) and SYPRO Ruby Protein Gel Stain from Molecular Probes (Leiden, The Netherlands). Protease-inhibitor cocktail tablets (“Complete EDTA-free”) were from Roche Molecular Biochemicals (Mannheim, Germany), and bovine serum albumin was from Serva Feinbiochemica (Heidelberg, Germany). All-*trans*-retinoic acid (RA), alkaline phosphatase from *Escherichia coli* (type III), cytosine β-D-arabinofuranoside, phenyl phosphate, phosphoserine, bicinchoninic acid kit for protein determination, and molecular-mass markers for SDS-polyacrylamide gel electrophoresis (PAGE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glutathione *S*-transferase (GST)-tagged fusion proteins containing amino acid residues 145–247 (SH2 domain) and 85–139 (SH3 domain) of Fyn were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Constructs encoding the GST-tagged fusion proteins containing amino acid residues 146–251 (SH2 domain) and 87–146 (SH3 domain) of Src [28] in pGEX-2TKX vector were obtained from Dr. V. Sovová (Institute of Molecular Genetics, Prague) and expressed in *Escherichia coli* strain BL21 by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The Src family selective tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3; negative control for inhibitor PP2) were obtained from Calbiochem (La Jolla, CA, USA). Stock solutions of PP2 and PP3 (10 mM) were prepared in dimethylsulfoxide. [γ-^{32}P]ATP (110 TBq/mmol; 370 MBq/ml) and glutathione Sepharose 4B were from Amersham Biosciences (Little Chalfont, England).

Cells

P19.X1 cells, a subclone of mouse EC cells P19 [29], were cultured in basic culture medium that was prepared by mixing Dulbecco’s modified Eagle’s medium and RPMI 1640 medium (l/l, vol/vol) containing 10% (v/v) fetal bovine serum, penicillin (l00 units/ml), and streptomycin (0.l mg/ml). Cells were grown at 37°C in 5% CO_2 in air and passaged every 2 days using 0.25% (w/v) trypsin/0.01% (w/v) EDTA in PBS, pH 7.5. Cell aggregates were formed by plating 10^5 cells/ml into bacteriological-grade Petri dishes. After 2 days, the cell aggregates were gently spun down, resuspended in a medium containing 10^{-6} M RA, and transferred into tissue culture dishes (diameter 6 cm) in aliquots of 10^5 cells/dish. Medium containing RA was changed every 2 days. In some cases, cells before extraction were incubated for 60 min at 37°C with Src family selective tyrosine kinase inhibitor PP2 or with PP3 (negative control for PP2) at a concentration 20 μM.

For immunofluorescence visualization on differentiated cells, a modified culturing procedure was applied [11] that made it possible to better discriminate neuron-like cells by eliminating proliferating nonneuronal cells. Briefly, cells in bacteriological-grade Petri dishes were aggregated 2 days in a medium containing 10^{-6} M RA. The aggregates were then dispersed into single cell suspension by treatment with 0.25% (w/v) trypsin/0.01% (w/v) EDTA in PBS, pH 7.5. The suspension (10^6 cells) in medium with RA was replated onto a culture dish (diameter 6 cm) containing poly-L-

lysine-coated glass coverslips. After 3 days, fresh medium with RA supplemented with cytosine arabinoside at concentration 5 μg/ml was added and culturing continued for an additional 4 days before fixation of the samples for immunofluorescence staining.

Antibodies

The following antibodies against protein tyrosine kinases were used: monoclonal antibody v-src (Ab-1) (IgG1, clone 327) against $pp60^{src}$ (Oncogene Research products, Boston, MA, USA); monoclonal antibody fyn (IgG2b, clone 25) against $p59^{fyn}$ (Transduction Laboratories, Lexington, KY, USA), and affinity-purified rabbit antibody Fyn (FYN3) against $p59^{fyn}$ (Santa Cruz Biotechnology). The monoclonal anti-phosphotyrosine antibody 4G10 (IgG1) and affinity-purified rabbit antibody against phosphotyrosine were from Upstate Laboratories (Lake Placid, NY, USA). The following monoclonal antibodies against tubulins were used: TU-31 (lgG2b) anti-peptide antibody prepared against the EYHAATRPDYISWGTQ peptide corresponding to human γ-tubulin sequence 434–449 [30], GTU-88 (IgG1) anti-peptide antibody prepared against the EEFA-TEGTDRKDVFFY peptide corresponding to human γ-tubulin sequence 38–53 (Sigma), TU-01 (lgG1) against α-tubulin (α-Tb) [31], TU-20 (IgG1) against neuron-specific class III β-tubulin [32], and 6-11B-1 (IgG2b) against acetylated α-tubulin (Acα-Tb; Sigma). For double-label immunofluorescence was used rabbit affinity-purified antibody TUB against αβ-tubulin dimer [33]. Actin was detected by rabbit affinity-purified antibody against the C-terminal peptide (Sigma). Monoclonal antibody NF-09 (IgG2a) against neurofilament protein NF-M [34] and rabbit antibody against nonmuscle myosin (Biomedical Technologies Inc., Stoughton, MA, USA) were used as negative controls in immunoprecipitation experiments. Rabbit antibody against GST was from Dr. Petr Dráber (Institute of Molecular Genetics, Prague, Czech Republic). Anti-mouse Ig antibodies and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI, USA); indocarbocyanate (Cy3)-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig antibody were from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

Preparation of cell extracts

Whole-cell extracts for SDS-PAGE were prepared as follows: cells on 6-cm Petri dishes were rinsed twice in cold MEM buffer (100 mM MES adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$), solubilized in hot SDS-sample buffer [35] without bromphenol blue (0.7 ml/dish), and boiled for 5 min.

When preparing the extract for immunoprecipitation and for binding to immobilized GST-fusion proteins, cells were rinsed twice in cold MEM buffer and extracted 10 min at 4°C with MEM buffer (0.6 ml/dish) supplemented with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), protease inhibitor cocktail, phosphatase inhibitors (1 mM Na3VO4, 1 mM NaF), and 1% (v/v) NP-40. The suspension was then spun down (20,000 × *g*, 15 min, 4°C), and supernatant collected.

Protein quantifications in lysates and SDS-PAGE-samples were performed, respectively, with bicinchoninic acid assay and silver dot assay [36] using bovine serum albumin as a standard.

Protein phosphatase treatment of extracts

For phosphatase treatment, 1% (v/v) NP-40 protein extracts were prepared in Tris buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$) supplemented with protease inhibitor cocktail. The extracts were incubated with alkaline phosphatase (1 unit/10 μg protein) or with buffer at 37°C for 60 min. The reaction was quenched by adding of the sample buffer for two-dimensional electrophoresis (2D-PAGE).

Immunoprecipitation

Immunoprecipitation was performed as described [37] using TBST (10 mM Tris–HCl adjusted to pH 7.4 with HCl, 150 mM NaCl, 0.05% (v/v) Tween 20) for dilution of extracts and for washings. For comparison of immunoprecipitation from unstimulated and stimulated cells, protein extracts from both stages were of the same protein concentration. Cell extracts were incubated with beads of protein A saturated with (I) rabbit antibody against kinase Fyn, (II) rabbit antibody against phosphotyrosine, (III) negative control rabbit antibody against nonmuscle myosin, (IV) mouse antibody TU-31 (IgG2b) against γ-tubulin, (V) negative control mouse antibody NF-09 (IgG2a), or with (VI) immobilized protein A alone. Alternatively, cell extracts were incubated with beads of protein L saturated with (I) mouse antibody against kinase Src (IgG1), (II) negative control mouse antibody NF-09 (IgG2a), and (III) immobilized protein L alone. Sedimented beads (30 μl) were incubated for 2 h at 4°C under constant shaking with 1 ml of the corresponding antibody in TBST. Antibodies against Src, Fyn, myosin, and phosphotyrosine were used at immunoglobulin concentration 4 μg/ml. Antibody TU-31 and control antibody NF-09 were prepared by mixing 0.4 ml of 10× concentrated supernatant with 0.8 ml of the TBST buffer. The beads were pelleted by centrifugation at 5000 × *g* for 1 min, washed four times (5 min each) in cold TBST, and incubated under rocking for 2 h at 4°C with 1 ml of cell extract, prepared by diluting the extract with TBST to final protein concentration 1.7 mg/ml. The beads were pelleted and washed four times (5 min each) in cold TBST, followed by boiling for 5 min in 100 μl of SDS-sample buffer to release the bound proteins. Alternatively, beads were washed twice in TBST and further processed in the kinase assay (see below).

Binding of cell extracts to GST fusion proteins

GST fusion proteins (5–15 μg) were non-covalently coupled to glutathione Sepharose beads (50 μl of sedimented beads) and used after washing in TBST for binding analysis. Sedimented beads were incubated under rocking for 1 h at 4°C with 0.5 ml of cell extract diluted with TBST to final concentration 1.7 mg/ml. Unbound material was removed by four washes in cold TBST, and bound proteins were eluted from the beads by boiling for 5 min in 80 μl of SDS-sample buffer. In competitive inhibition experiments, phenyl phosphate or phosphoserine was added to cell extracts at concentrations of 4.5 or 45 mM before adding the beads, and the mixtures were incubated for 1 h followed by washing and elution as indicated above.

In vitro kinase assay

Beads with immunoprecipitated material were washed twice in kinase buffer (25 mM HEPES adjusted to pH 7.2 with NaOH, 3 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM NaF, 0.1% (v/v) NP-40), and pelleted beads were resuspended in 30 μl of kinase buffer supplemented with 185 kBq of [γ-^{32}P]ATP. After incubation for 15 min at 37°C, the reaction was stopped by washing the beads three times in kinase buffer and the labeled immunocomplexes were solubilized by boiling for 5 min in 80 μl of SDS-sample buffer. The samples (10 μl) were resolved by SDS-PAGE, transferred to nitrocellulose, and the ^{32}P-labeled proteins were detected by autoradiography. Alternatively, the labeled immunocomplexes were solubilized in 80 μl of sample buffer for 2D-PAGE, and 70-μl aliquots were used for 2D-PAGE. After electrotransfer to nitrocellulose, the ^{32}P-labeled proteins were detected by autoradiography and blots were then immunostained.

Gel electrophoresis and immunoblotting

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% or 10% gels, electrophoretic transfer of separated proteins onto nitrocellulose, and details of the immunostaining procedure are described elsewhere [38]. SDS-sample buffer was supplemented with 0.2 mM Na_3V0_4. The two-dimensional electrophoresis (2D-PAGE) was performed essentially as described [39]. The unlabeled samples containing about 30 μg protein were diluted with sample buffer containing 7 M urea, 2 M thiourea, 20 mM Tris, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) Triton X-100, 1% (w/v) DL-dithiothreitol, and 2% (v/v) IPG buffer, pH 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden). After kinase assay Immobiline DryStrip gels with linear pH 4–7 gradient, 7 or 11 cm long (Amersham Pharmacia Biotech), were rehydrated using 250 μl of prepared sample (in IPG sample buffer). Each strip was overlaid with mineral oil and left overnight at room temperature. Strips were focused for a total of 22 kV h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed on 8.5% SDS-PAGE, and separated proteins were transferred onto nitrocellulose by electroblotting. The anti-tubulin monoclonal antibodies TU-01, TU-20, and TU-32, in the form of spent culture supernatants, were used undiluted, whereas the antibodies 6-11B-1 and GTU-88 were diluted 1:2000 and 1:5000, respectively. Monoclonal antibodies against kinase Src, kinase Fyn, and phosphotyrosine were diluted 1:300, 1:150, and 1:1500, respectively. Rabbit antibody against actin was diluted 1:200, and rabbit antibody against GST 1:30,000. Bound antibodies were detected after incubation of the blots with secondary antibodies diluted 1:10,000 and after washing with chemiluminescence reagents in accordance with the manufacturer's directions. Exposed autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY, USA) were quantified by densitometry using gel documentation system GDS 7500 and GelBase/GelBlot Pro analysis software (UVP, Upland, CA, USA).

Immunofluorescence

Immunofluorescence microscopy was performed on fixed cells as described [37]. Shortly, cells were rinsed briefly with microtubule-stabilizing buffer (MSB; MEM buffer supplemented with 4% (w/v) polyethylene glycol 6000), extracted for 1 min with 0.2% Triton X-100, and fixed for 20 min in 3% formaldehyde in MSB. Preparations were incubated for 45 min at room temperature with primary antibodies. Polyclonal anti-tubulin antibody TUB was diluted 1:5, TU-20 monoclonal antibody against neuron-specific class III β-tubulin was used as undiluted supernatant, and GTU-88 monoclonal antibody against γ-tubulin was diluted 1:1000. Cy3-conjugated anti-mouse Ig antibody and FITC-conjugated anti-rabbit Ig antibody were diluted 1:500 and 1:200, respectively. For double-label immunofluorescence staining, the coverslips were incubated simultaneously with TU-20 and polyclonal anti-tubulin antibody, and after washing were incubated simultaneously with the secondary fluorochrome-conjugated antibodies. The preparations were mounted in MOWIOL 4–88 (Calbiochem AG, Lucerne, Switzerland) and examined with Olympus A70 Provis microscope equipped with 60× and 40× water-immersion objectives. Images were recorded with a Life Science Resources KAF 1400 cooled CCD camera. Conjugates alone did not give any detectable staining.

Results

Expression of kinases and tubulins in differentiating P19 cells

To compare the expression of protein tyrosine kinases of Src family and tubulins during neuronal differentiation of P19 cells, blots of whole cell extracts from unstimulated cells and cells stimulated with RA for 1–12 days were

probed with antibodies against kinases Src and Fyn and with antibodies against tubulins. Neuron-specific class III β-tubulin was used as a marker of neuronal differentiation and acetylated α-tubulin as a marker of stable microtubules in newly formed neuronal projections. Typical results are shown in Fig. 1A. Day 0 stands for cells that were aggregated but not incubated in the presence of RA. The same amount of proteins was loaded at each time interval. An increased expression of Src, Fyn, and acetylated α-tubulin appeared after 48-h incubation of cells with RA and reached its maximum after 12 days. Densitometric measurements of immunoblots showed that within the time interval between day 0 and day 12, the amounts of Src kinase increased approximately six times, Fyn 11 times and acetylated α-tubulin 16 times. The class III β-tubulin was not detected in unstimulated cells and began to appear after 3 days of incubation with RA. At the same time, the amount of α-tubulins rose only moderately. The used TU-01 anti-tubulin antibody reacts with an epitope located in the conservative N-terminal region and recognizes all α-tubulin isotype classes and their posttranslational modifications [31]. The amount of detected γ-tubulin did not change during differentiation and the same was true for actin (not shown). Elevated expression of protein tyrosine kinases during neuronal differentiation corresponded with the increased level of proteins phosphorylated on tyrosine (Fig. 1B). Both qualitative and quantitative differences were observed between unstimulated and stimulated cells when stained with the monoclonal antibody 4G10 against phosphorylated tyrosine.

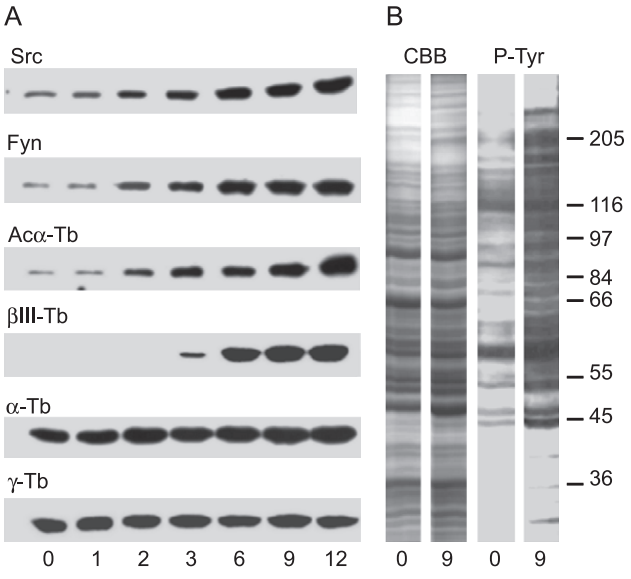

Fig. 1. Immunoblot analysis of whole cell extracts from unstimulated cells (day 0) and cells stimulated with RA for 1–12 days. (A) Immunostaining with antibodies against kinase Src (Src), kinase Fyn (Fyn), acetylated α-tubulin (Acα-Tb), neuron-specific β-tubulin class III (βIII-Tb), α-tubulin (α-Tb), and γ-tubulin (γ-Tb). (B) Coomassie blue staining (CBB) and immunostaining with anti-phosphotyrosine antibody (P-Tyr). Numbers 0–12 indicate days of incubation of cells with RA. The same amount of proteins (10μg) was loaded into each lane. Molecular mass markers (in kDa) are indicated to the left of B.

Stimulation of P19 cells with RA was accompanied with substantial changes in cell morphology and in distribution of γ-tubulin. Double-label immunostaining with polyclonal antibody against αβ-tubulin dimer and monoclonal antibody against neuron-specific class III β-tubulin proved that neuron-specific tubulin was absent in unstimulated cells (Figs. 2A and B), but was detectable in neurite projections of neuron-like cells after 9 days stimulation with RA (Figs. 2C and D). Immunofluorescence staining with anti-γ-tubulin antibody GTU-88 revealed that γ-tubulin in unstimulated cells was accumulated in MTOC, but diffuse staining was also observed (Fig. 2E). In stimulated cells, a dot-like staining was found both in cell bodies and in neurite projections (Fig. 2F). A similar staining was obtained with TU-31 antibody.

γ-Tubulin is phosphorylated in vivo

Previous studies using 2D-PAGE revealed in brain tissue multiple isoforms of γ-tubulin. We therefore investigated whether γ-tubulin in P19 cells stimulated to neuronal differentiation was also modified in vivo. Two-dimensional analysis of 1% NP-40 extracts followed by immunoblotting analysis of separated proteins with anti-γ-tubulin antibody GTU-88 confirmed that soluble forms of γ-tubulin did exist in multiple isoforms. As shown in Fig. 3 (upper panel), multiple isoforms were detectable, and the isoelectric point of the major γ-tubulin isoform was 6.00. Repeated experiments with varying protein loadings and with different exposure times of autoradiography films revealed the presence of five isoforms. To determine whether γ-tubulin is modified by phosphorylation, the extracts were treated with alkaline phosphatase. Under these conditions, the two most acidic isoforms were not observed (Fig. 3, lower panel). The decrease in isoform number was not observed in samples incubated without phosphatase (not shown). These data document that γ-tubulin is phosphorylated in vivo.

Kinases form complexes with γ-tubulin

Immunoprecipitation experiments showed that γ-tubulin could be precipitated from resting and stimulated cells specifically with monoclonal anti-Src antibody (IgG1) immobilized on protein L (Fig. 4A, panel "γ-Tb", lanes 1 and 3). Stimulation enhanced the amount of precipitated material. No staining in the position of γ-tubulin was observed when the immobilized anti-Src antibody was incubated without the extract (Fig. 4A, panel "γ-Tb", lane 2), when the negative control antibody NF-09 (IgG2a) was used for immunoprecipitation of extract from stimulated cells (Fig. 4A, panel "γ-Tb", lane 4), or when protein L without the antibody was incubated with extract from stimulated cells (Fig. 4A, panel "γ-Tb", lane 5). Staining of the precipitated material with anti-Src antibody confirmed

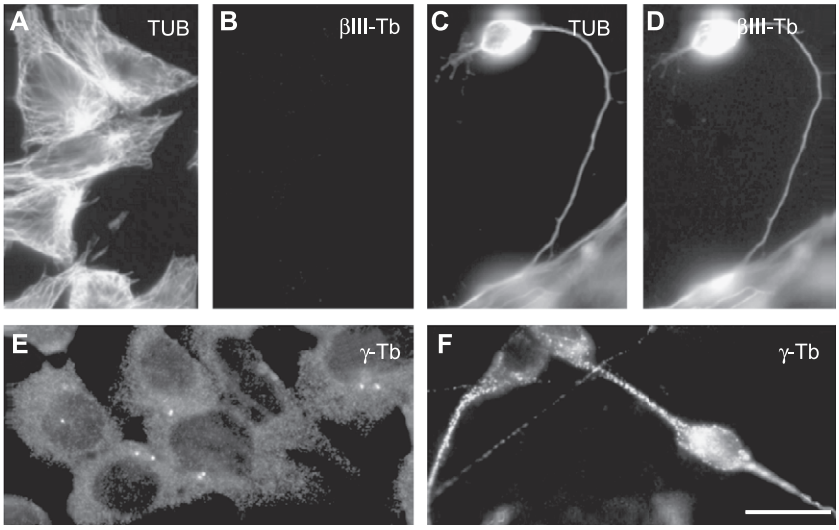

Fig. 2. Immunofluorescence localization of tubulins in resting and activated P19 cells. Staining of unstimulated cells (A, B, and E) and cells stimulated with RA for 9 days (C, D, and F). Double-label staining with polyclonal antibody (TUB) against αβ-tubulin dimers (A and C) and monoclonal antibody (βIII-Tb) against neuron-specific β-tubulin class III (B and D). Each pair (A–B and C–D) represents the same cells. Distribution of γ-tubulin (γ-Tb) is shown in E and F. Cells were extracted with Triton X-100 and fixed in formaldehyde. Scale bar, 20 μm. Comparable magnifications are in A–F.

the presence of Src kinase in unstimulated and stimulated cells (Fig. 4A, panel "Src", lanes 1 and 3). Staining with anti-phosphotyrosine antibody revealed that protein phosphorylated on tyrosine abounded in a position corresponding to relative electrophoretic mobility of Src kinase in both unstimulated and stimulated cells (Fig. 4A, panel "P-Tyr, lanes 1 and 3). No co-precipitation of Fyn was observed (not shown).

A similar set of immunoprecipitation experiments with rabbit anti-Fyn antibody immobilized on protein A is shown in Fig. 4B. γ-Tubulin could be precipitated specifically from resting and stimulated cells (Fig. 4B, panel "γ-Tb", lanes 1 and 3). But again, the amount of precipitated γ-tubulin was higher if stimulated cells were used. No staining in the position of γ-tubulin was observed when immobilized anti-Fyn antibody was incubated without the extract (Fig. 4B, panel "γ-Tb", lane 2), when rabbit negative control anti-

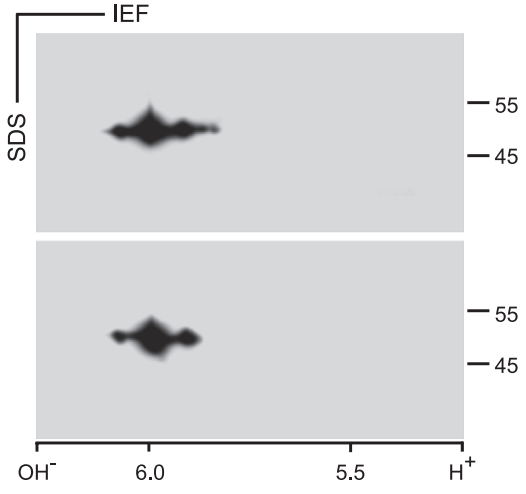

Fig. 3. Immunoblot analysis of extracts from cells stimulated with RA for 9 days after two-dimensional electrophoresis. Extracted proteins (upper panel) or extracted proteins treated with alkaline phosphatase were separated by 2D-PAGE and immunoblotted with anti-(γ-tubulin) antibody GTU-88. Molecular mass markers (in kDa) are indicated on the right. The pI scale is shown along the bottom of the figure. IEF, isoelectric focusing.

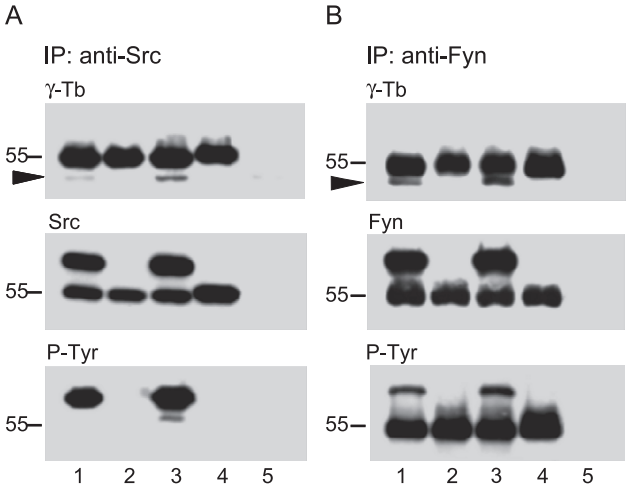

Fig. 4. Immunoprecipitation of extracts from resting and activated P19 cells with anti-(Src) and anti-(Fyn) antibodies. Samples were precipitated with mouse monoclonal anti-(Src) antibody bound to immobilized Protein L (A) or with rabbit anti-(Fyn) antibody bound to immobilized Protein A (B). Immunoprecipitation from unstimulated cells (lane 1) or cells stimulated with RA for 9 days (lanes 3–5). Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), kinase Src (Src), kinase Fyn (Fyn), and phosphotyrosine (P-Tyr). Proteins immunoprecipitated from unstimulated cells (lane 1), immobilized immunoglobulin not incubated with cell extract (lane 2), proteins immunoprecipitated from stimulated cells (lane 3), proteins immunoprecipitated from stimulated cells with negative control antibody (lane 4), and proteins from stimulated cells bound to carrier without antibody (lane 5). Molecular mass markers (in kDa) are indicated to the left. Arrowheads indicate the position of γ-tubulin.

body against myosin was used for immunoprecipitation of extract from stimulated cells (Fig. 4B, panel "γ-Tb", lane 4), or when protein A without the antibody was incubated with extract from stimulated cells (Fig. 4B, panel "γ-Tb", lane 5). Staining of precipitated material with anti-Fyn antibody confirmed the presence of Fyn kinase in unstimulated and stimulated cells (Fig. 4B, panel "Fyn", lanes 1 and 3). Staining with anti-phosphotyrosine antibody showed that the protein phosphorylated on tyrosine was present in a position corresponding to relative electrophoretic mobility of Fyn kinase in both unstimulated and stimulated cells (Fig. 4B, panel "P-Tyr, lanes 1 and 3). No co-precipitation of Src was observed (not shown). When samples precipitated from stimulated cells with anti-Src or anti-Fyn antibodies were separated by SDS-PAGE and gels were subsequently stained with sensitive SYPRO Ruby Protein Gel Stain, no other proteins were detectable except for immunoglobulin heavy and light chains.

Because antibodies against Src or Fyn kinase co-precipitated larger amounts of γ-tubulin from stimulated cells than from unstimulated cells, and because tyrosine-phosphorylated forms of kinases were present in such complexes, precipitation with rabbit anti-phosphotyrosine antibody bound to immobilized Protein A was carried out. The resulting precipitates contained γ-tubulin (Fig. 5A, panel "γ-Tb", lane 1), kinase Src (Fig. 5A, panel "Src", lane 1), and kinase Fyn (Fig. 5A, panel "Fyn", lane 1). No staining in corresponding positions was observed when rabbit negative control antibody against myosin was used for immunoprecipitation or when protein A without the antibody was incubated with cell extract.

To determine whether proteins phosphorylated by Src kinases are involved in the formation of γ-tubulin complexes, precipitation with anti-protein phosphorylated on tyrosine (P-Tyr) antibody was performed in the presence of Src family selective tyrosine kinase inhibitor PP2. As control, cells untreated with PP2 or cells treated with negative control inhibitor PP3 were used. In PP2-treated cells, the amount of co-precipitated γ-tubulin was smaller (Fig. 5B, panel "γ-Tb", lane 2) than in untreated cells (Fig. 5B, panel "γ-Tb", lane 1) or cells treated with PP3 (Fig. 5B, panel "γ-Tb", lane 3). Staining with anti-phosphotyrosine antibody showed a substantially lower level of proteins phosphorylated on tyrosine in PP2-treated cells (Fig. 5B, panel "P-Tyr", lane 2) than in untreated cells (Fig. 5B, panel "P-Tyr", lane 1) or in cells treated with PP3 (Fig. 5B, panel "P-Tyr", lane 3). The possibility of an unspecific attachment of cytoplasmic proteins to complexes containing γ-tubulin can be eliminated because no actin was detectable in the immunoprecipitated material with anti-actin antibody (not shown).

Combined data from immunoprecipitation experiments indicate that soluble γ-tubulin in P19 cells appears in complexes with protein tyrosine kinases of Src family. A larger amount of γ-tubulin is present in complexes from stimulated cells, with higher expression of kinases Src and Fyn.

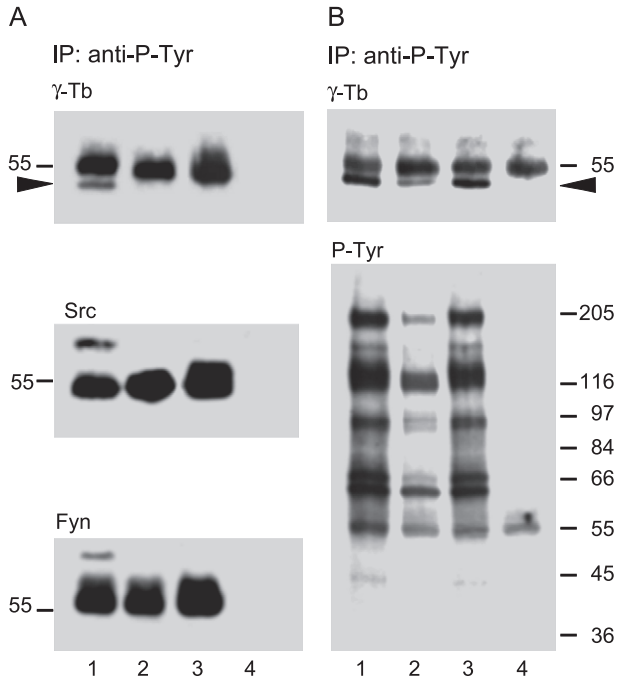

Fig. 5. Immunoprecipitation of extracts from activated P19 cells with anti-(phosphotyrosine) antibody. Samples from cells stimulated with RA for 9 days (A) or from stimulated cells treated with Src family selective inhibitor PP2 (B) were precipitated with rabbit anti-phosphotyrosine antibody bound to immobilized Protein A. Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), kinase Src (Src), kinase Fyn (Fyn), and phosphotyrosine (P-Tyr). (A) Immunoprecipitated proteins (lane 1), immobilized immunoglobulin not incubated with cell extract (lane 2), proteins immunoprecipitated from cells with negative control antibody (lane 3), and proteins bound to carrier without antibody (lane 4). (B) Proteins precipitated from untreated cells (lane 1), from PP2-treated cells (lane 2), and from PP3-treated cells (lane 3), and immobilized immunoglobulin not incubated with cell extract (lane 4). Molecular mass markers (in kDa) are indicated to the left in A and to the right in B. Arrowheads indicate the position of γ-tubulin.

γ-Tubulin complexes have kinase activity dependent on Src kinases

A possible association of γ-tubulin with kinase substrates was examined with the immunocomplex kinase assay. Stimulated cells were precipitated with monoclonal anti-(γ-tubulin) antibody TU-31 or with negative control monoclonal antibody NF-09 immobilized on protein A. Immunocomplexes were then subjected to the in vitro kinase assay, and proteins separated by SDS-PAGE or 2D-PAGE were electroblotted and visualized by autoradiography. γ-Tubulin in the extract from stimulated cells was associated with several kinase substrates (Fig. 6A, lane 1), whereas no kinase activity was detected after precipitation with the control antibody (Fig. 6A, lane 3). When the cells were cultured in the presence of Src family selective tyrosine kinase inhibitor PP2 before precipitation, a lower level of phosphorylation was clearly detected in proteins with relative molecular weights of approximately 75, 65, 60, and 50 kDa (Fig. 6A, lane 2). These proteins are thus substrates for

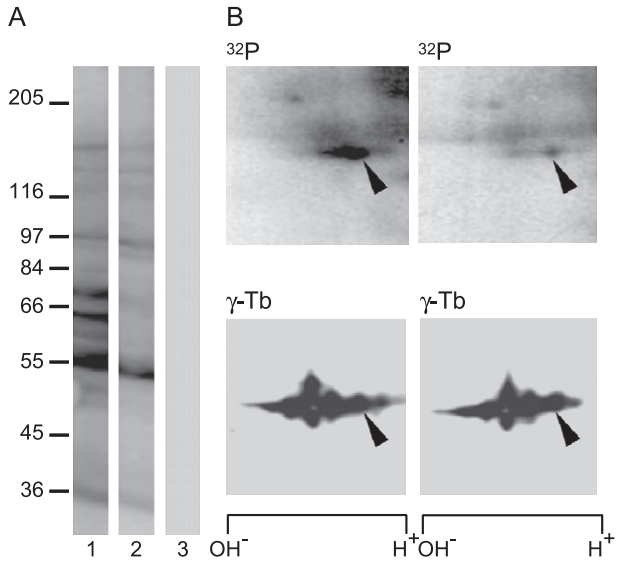

Fig. 6. The kinase activity of immunocomplexes containing γ-tubulin. Samples from cells stimulated with RA for 9 days or from stimulated cells treated with Src family selective inhibitor PP2 were precipitated with anti-(γ-tubulin) antibody TU-31 or negative control antibody NF-09 bound to immobilized Protein A. Immunocomplexes were subjected to in vitro kinase assay and electrophoretically separated. (A) Autoradiography after SDS-PAGE. Precipitation with TU-31 from untreated (lane 1) or PP2-treated cells (lane 2). Precipitation with NF-09 from untreated cells (lane 3). Molecular mass markers (in kDa) are indicated to the left. (B) Autoradiography (^{32}P) and immunoblotting with anti-(γ-tubulin) antibody GTU-88 (γ-Tb) after 2D-PAGE. Precipitation with TU-31 from untreated (left column) or PP2-treated cells (right column). Arrowheads indicate the same positions.

Src family kinases. When solubilized labeled immunocomplexes were analyzed by 2D-PAGE, autoradiography revealed a signal in the region of acidic γ-tubulin isoforms. This was confirmed by staining the same blot with anti-γ-tubulin antibody GTU-88 (Fig. 6B, left panels "^{32}P" and "γ-Tb"). When the cells were treated with the PP2 inhibitor, a weaker autoradiography signal (Fig. 6B, right panel "^{32}P") was detected in the region of acidic γ-tubulin isoforms (Fig. 6B, right panel "γ-Tb"). These data demonstrate that active Src family kinases are part of γ-tubulin complexes and that γ-tubulin could be a substrate for Src family kinases.

γ-Tubulin complexes bind to SH2 domains of kinases

Guided by the fact that there is an association of γ-tubulin with active Src family kinases, we investigated whether the SH2 or SH3 kinase domains, which are important in the assembly of protein complexes, participate in this interaction. We have performed in vitro binding experiments with immobilized GST fusion proteins containing the corresponding domains of Src and Fyn kinases. γ-Tubulin bound to GST-Fyn-SH2 and GST-Src-SH2 fusion proteins, but not to GST-Fyn-SH3 and GST-Src-SH3 fusion proteins or GST alone. Under identical conditions, more γ-tubulin was bound to GST-Fyn-SH2 than to GST-Src-SH2 (Fig. 7A, panel "γ-Tb"). Similarly, proteins phosphorylated on tyrosine were also bound only to GST-Fyn-SH2 and GST-Src-SH2 fusion proteins, and more proteins phosphorylated on tyrosine were bound to GST-Fyn-SH2 (Fig. 7A, panel "P-Tyr"). The amount of immobilized fusion proteins is shown by staining with anti-GST antibody (Fig. 7A, panel "GST"). Anti-actin antibody did not detect any binding of actin to SH2 or SH3 domains of kinases.

To determine whether the observed interaction of γ-tubulin with SH2 domains of kinases is an SH2-phosphotyrosin type interaction, we performed a competition experiment with phenyl phosphate, an analogue of phosphotyrosine. Phenyl phosphate (45 mM) inhibited the binding of γ-tubulin to GST-Fyn-SH2 and GST-Src-SH2

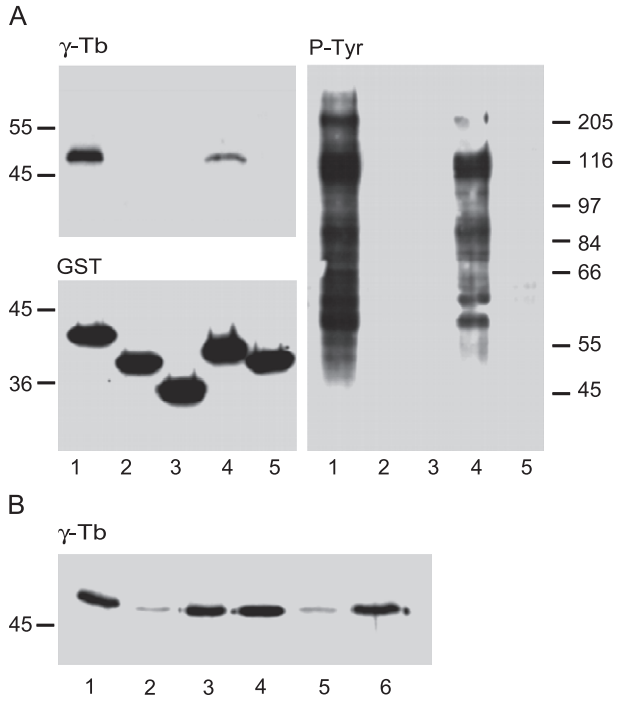

Fig. 7. Binding of γ-tubulin to SH2 domains of Fyn and Src kinases. Samples from cells stimulated with RA for 9 days were incubated with GST-fusion proteins or GST alone (negative control) immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), GST (GST), or phosphotyrosine (P-Tyr). (A) Comparison of γ-tubulin binding to SH2 and SH3 domains of kinases Src and Fyn. GST-SH2 domain of Fyn kinase (lane 1), GST-SH3 domain of Fyn kinase (lane 2), GST alone (lane 3), GST-SH2 domain of Src kinase (lane 4), and GST-SH3 domain of Src kinase (lane 5). Fusion proteins (10 μg) were immobilized to Sepharose beads. (B) Effect of phenyl phosphate and phosphoserine on the binding of γ-tubulin to SH2 domains of kinases Src and Fyn. GST-SH2 domain of Fyn kinase (lanes 1–3) and GST-SH2 domain of Src kinase (lanes 4–6) were incubated with cell extract in the absence (lanes 1 and 4) or presence of 45 mM phenyl phosphate (lanes 2 and 5) or 45 mM phosphoserine (lanes 3 and 6). GST-SH2 domain (5 μg) of Fyn kinase or 15 μg of GST-SH2 domain of Src kinase was immobilized to Sepharose beads. Molecular mass markers (in kDa) are indicated on the left in panels γ-Tb and GST, and on the right in panel P-Tyr.

fusion proteins as can be seen in Fig. 7B (lanes 2 and 5). With GST-Src-SH2, 4.5 mM phenyl phosphate was also tested; it still had some inhibitory effect on γ-tubulin binding (not shown). This inhibition was specific, because the same concentrations of phosphoserine had no effect on binding of γ-tubulin to SH2 domains of kinases (Fig. 7B, lanes 3 and 6).

Discussion

Quantitative immunoblotting of P19 cells stimulated with retinoic acid revealed that the expression of Src family kinases Src and Fyn was substantially increased during neuronal differentiation documented by typical changes in cell morphology. Elevated expression of kinases corresponded with the increased level of proteins phosphorylated on tyrosine. Although the level of γ-tubulin did not vary during differentiation, substantial changes were observed in its subcellular localization. Immunofluorescence microscopy revealed in stimulated cells an association of γ-tubulin with detergent-resistant fraction in cell bodies and neurite projections. Because the greater part of γ-tubulin in animal cells is not associated with polymerized microtubules, its binding to the fraction not extractable by detergent indicates an association with intracellular membranous components under the extraction conditions used (0.2% Triton X-100 in MSB, at 37°C). A significant association of γ-tubulin with intracellular components was observed in various cell lines.

Immunoprecipitation experiments with antibodies against Src kinase, Fyn kinase, and phosphotyrosine revealed that γ-tubulin in stimulated cells was associated with kinases. Binding experiments with recombinant SH2 and SH3 domains of these kinases revealed that γ-tubulin or protein complexes containing γ-tubulin bound to SH2 domains, and that this interaction was of the SH2-phosphotyrosine type. The presence of Src family kinases in complexes with γ-tubulin was also confirmed by the in vitro kinase assay with material precipitated with anti-γ-tubulin antibody from stimulated P19 cells, which were either treated or not with Src family selective inhibitor PP2. As the Fyn kinase did not co-precipitate with Src kinase and, reciprocally, the Src kinase did not co-precipitate with Fyn kinase, it is probable that the two kinases form different types of complexes with γ-tubulin. Sucrose gradient centrifugation, which effectively separates γTuRC from γTuSC in P19 cells, revealed that kinases Src and Fyn did not associate with γTuRC (L. Macùrek, unpublished). In contrast, the serine–threonine polo-like kinase (Plk) that plays an important role in the regulation of cell division co-eluted with γ-tubulin during gel filtration on Superose 6 column in the range of large complexes. Plk also phosphorylated γ-tubulin [40]. Moreover, it has been reported that lipid phosphoinositide 3-kinase (PI 3-kinase) binds to γ-tubulin in response to insulin [41], and that the 55-kDa regulatory subunit of PI 3-kinase interacts with γ-tubulin [42]. An association of protein tyrosine kinase p53/p56^{lyn}, another member of the Src family kinases, with γ-tubulin was reported in rat basophilic leukemia cells [27]. Collectively, these data suggest that γ-tubulin could participate in the formation of protein complexes whose activity might be regulated by phosphorylation.

While there are only two functional γ-tubulin genes in mammals [43], multiple isoelectric variants of γ-tubulin were detected by two-dimensional electrophoresis in differentiated P19 cells. γ-Tubulin charge variants were already described in brain [22,44], nucleated erythrocytes [45], and budding yeast [26]. In the last case, phosphorylation of the γ-tubulin residue Tyr 445, which is invariably present in all γ-tubulins, was described [26]. Mutation of this residue changed the microtubule dynamics, and it was suggested that γ-tubulin could play a role in the dynamics of the plus ends of microtubules [26,46]. In vitro kinase assay with material precipitated with anti-(γ-tubulin) antibody followed by 2D-PAGE revealed the presence of labeled protein in the position of the acidic variant of γ-tubulin. The labeling was weaker when cells were pretreated with an inhibitor of Src kinases. This indicates that γ-tubulin could be the substrate for Src family kinases. When monoclonal anti-phosphotyrosine antibody was used for probing the blotted proteins after two-dimensional electrophoresis, staining in the position of acidic γ-tubulin isoforms was detectable, even though it was weak (V. Sulimenko unpublished). On the other hand, when polyclonal or monoclonal anti-phosphotyrosine antibodies were used for precipitation of differentiated P19 cells, γ-tubulin was present in immunocomplexes, but no accumulation of protein phosphorylated on tyrosine was detected in the position of γ-tubulin. This suggests that only a small fraction of γ-tubulin is phosphorylated on tyrosine in activated P19 cells compared to other co-precipitated proteins.

In conclusion, the presented data demonstrate for the first time that in P19 cells induced to neuronal differentiation, the Src family protein tyrosine kinases Src and Fyn form complexes with γ-tubulin by an interaction with their SH2 domains. Activity of these kinases might regulate the interaction of γ-tubulin with tubulin dimers or other proteins during neurogenesis.

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