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Regulation of microtubule nucleation from membranes by complexes of membrane-bound γ -tubulin with Fyn kinase and phosphoinositide 3-kinase

Libor MACUREK^{*}, Eduarda DRÁBEROVÁ^{*}, Věra RICHTEROVÁ^{*}, Vadym SULIMENKO^{*}, Tetyana SULIMENKO^{*}, Lubica DRÁBEROVÁ[†], Vladimíra MARKOVÁ^{*}, and Pavel DRÁBER^{*1}

^{*}Department of Biology of Cytoskeleton and [†]Department of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic

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¹Corresponding author: Dr. Pavel Dráber, Department of Biology of Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic.

Tel.:(420) 241 062 632; Fax: (420) 241 062 758

E-mail: paveldra@img.cas.cz

ABSTRACT

Molecular mechanisms controlling microtubule formation in cells with non-centrosomal microtubular arrays are not yet fully understood. The key component of microtubule nucleation is γ -tubulin. Although previous data suggested that tyrosine kinases might serve as regulators of γ -tubulin function, their exact roles remain enigmatic. Here we show that a pool of γ -tubulin associates with detergent-resistant membranes in differentiating P19 embryonal carcinoma cells which exhibit elevated expression of Src family kinase Fyn. Microtubule assembly assays demonstrated that membrane-associated γ -tubulin complexes are capable of initiating formation of microtubules. Pretreatment of the cells with Src family kinase inhibitors or wortmannin blocked the nucleation activity of γ -tubulin complexes. Immunoprecipitation experiments revealed that membrane-associated γ -tubulin forms complexes with Fyn kinase and phosphoinositide 3-kinase (PI3K). Furthermore, in vitro kinase assays showed that p85 α regulatory subunit of PI3K serves as Fyn kinase substrate. Direct interaction of γ -tubulin with C-terminal Src homology 2 domain of p85 α was determined by pull-down experiments and immunoprecipitation experiments with cells expressing truncated forms of p85 α . The combined data suggest that Fyn kinase and PI3K might take a part in modulation of membrane-associated γ -tubulin activities.

Key words: Fyn kinase, detergent-resistant membranes, phosphoinositide 3-kinase (PI3K), PI3K regulatory subunit (p85 α), γ -tubulin, SH2-domain

Abbreviations used: Ab, antibody; DRM, detergent-resistant membrane; GCP2, γ -tubulin complex protein 2; Fyn, protein tyrosine kinase p59^{Fyn}; mAb, mouse monoclonal antibody; MTOC, microtubule organizing center; p85 α , regulatory p85 α subunit of PI3K; PI3K, phosphoinositide 3-kinase; RA, *all-trans*-retinoic acid; SH2 and SH3, Src homology 2 and 3 domains.

INTRODUCTION

In most proliferating and migrating animal cells, centrosome is the main site for microtubule nucleation leading to the formation of radial microtubules. In contrast, differentiated cell types including muscle, epithelial and neuronal cells possess non-radial microtubules arranged in non-centrosomal arrays [1]. In these cell types, the mechanism of microtubule nucleation is not fully understood. One of the key components required for microtubule organization is γ -tubulin [2]. The γ -tubulin is associated with other proteins in cytoplasmic complexes. Large γ -tubulin ring complexes (γ TuRCs) are formed by γ -tubulin small complexes (γ TuSCs), comprising two molecules of γ -tubulin and one molecule each of γ -tubulin complex proteins (GCP)2 and GCP3, beside some other proteins [3]. These complexes are usually embedded into microtubule organizing centers (MTOCs), where they nucleate microtubules. We and others have demonstrated that γ -tubulin is also associated with cellular membranes [4,5] and that protein tyrosin kinases form complexes with γ -tubulin [6,7]. Yet it remains to be clarified what are the regulatory mechanisms of microtubule nucleation from membranes.

Non-receptor protein tyrosin kinase p59^{fyn} (Fyn), a member of the Src kinases family, has been implicated in events regulating neuronal differentiation and function of neuronal cells. It is highly expressed in developing neurons, and the Fyn knock-out mice exhibit defects in neurite outgrowth in response to cell adhesion molecules. On the other hand, knock-outs of other kinases of Src family, the Src and Yes, do not lead to obvious defects in brain development [8]. Fyn therefore seems to play a key role in neuronal differentiation. It is recruited in neuronal cells to lipid microdomains rich in cholesterol and sphingolipids, and forms functional complexes with transmembrane proteins and other signaling molecules [9].

It has been shown that Src and Lck from the family of Src kinases can also be involved in the regulation of phosphoinositide 3-kinase (PI3K)-dependent signaling pathways [10] required for neurite outgrowth [11]. Class IA PI3Ks (hereafter referred to as PI3K) are heterodimers of a p110 catalytic subunit and a regulatory subunit. The most extensively studied regulatory subunit is the p85 α . It consists of an inter-Src-homology 2 (SH2) domain flanked by two SH3 domains. The SH3 domain and RhoGAP domain reside in the N-terminal region of the molecule. Upon stimulation, the p85 α binds to phosphorylated receptors or adaptor proteins, and the bond relieves basal inhibition of the catalytic subunit by p85 α [12]. Although complexes of p85 α with γ -tubulin has been described [13,14], the location of γ -tubulin binding site on p85 α molecule as well as the type of this interaction are unknown. It is also unclear what is the biological relevance of γ -tubulin-p85 α association.

Murine P19 embryonal carcinoma cells offer a well-characterized experimental model for *in vitro* neuronal differentiation induced by treatment with all-*trans*-retinoic acid (RA) [15]. RA-stimulation induces changes in the expression signaling molecules [16] that make these cells suitable for biochemical studies of early stages of neuronal differentiation. We have previously shown that RA-induced differentiation of P19 cells results in an enhanced expression of Fyn, and that an association of γ -tubulin with detergent-resistant patches is detectable with immunofluorescence microscopy. Moreover, γ -tubulin forms complexes with Fyn and tyrosine-phosphorylated proteins [16]. To address the hypothesis that Src family kinases could be involved in the regulation of non-centrosomal microtubule formation, we characterized the membrane-associated γ -tubulin complexes in differentiating P19 cells.

MATERIAL AND METHODS

Materials

Protease-inhibitor cocktail tablets were from Roche. Src-family tyrosine kinase inhibitor PP2 and PP3 (negative control for PP2) were obtained from Calbiochem. RA, cytosine β -D-arabinofuranoside, saponin, methyl- β -cyclodextrin, wortmannin and the Src-family tyrosine kinase inhibitor SU6656 were from Sigma. Imatinib mesylate, inhibitor of tyrosine kinases Bcr-Abl and c-kit was kindly donated by Novartis. 10 mM stock solutions of wortmannin, SU6656, PP2, PP3 and imatinib mesylate were prepared in DMSO. Recombinant PI3K (p110 δ complexed to p85 α) and Fyn were bought from Upstate Laboratories. Tubulin was prepared from porcine brain [17].

DNA constructs

Total RNA was isolated from BALB/c adult mouse brain using Trizol reagent (Gibco-BRL). Reverse transcription was performed with random hexamers and SuperScript III Reverse Transcriptase kit (Invitrogen). The fragment coding mouse GCP2 (GenBank NM_133755) was amplified by PCR using forward 5'-AGTCGGATCCAGCGAATTTTCGGATTAC-3' and reverse 5'-AAGCGTCTCGACTCAGCCAATCAGGAAATCTC-3' primers and total cDNA as template. Isolated fragment was ligated into pGEX-6P-1 vector (Amersham Biosciences) for preparation of GST-tagged fusion protein.

Full-length cDNA of human p85 α was a gift of Dr. L. Rameh [18]. Restriction sites were introduced by PCR, and p85 α fragments were subcloned into pGEX-6P-1. The fragment encoding the cSH2 domain of p85 α carrying R649L mutation in FLVR motif was amplified from p85-CT R649L construct provided by Dr. T. O. Chan [10] and subcloned into pGEX-6P-1 (cSH2-R649L). For *in vivo* experiments, p85 α and its fragments were subcloned into pCI-NEO (Promega) expression vector. The p85 α -CTwt construct encoding the C-terminal fragment of p85 α was provided by Dr. T. O. Chan. Full length cDNA of mouse Fyn [19] was obtained from Dr. Pe. Dráber. Construct pFYN-EGFP was prepared by subcloning of Fyn into pEGFP-N3 vector (Clontech).

Full-length cDNA of human γ -tubulin was obtained from Dr. B. Oakley [20]. Restriction sites *EcoRI* and *XhoI* were introduced by PCR, and full-length γ -tubulin was subcloned into pGEX-6P-1 vector for production of fusion protein. Constructs were verified by restriction mapping and sequencing. GST-tagged fusion proteins were expressed in *Escherichia coli* strain BL-21 after IPTG induction and purified on Glutathione Sepharose 4B Fast Flow beads (Amersham) according to manufacturer's directions.

Antibodies

Rabbit antibody (Ab) and mouse monoclonal Ab (mAb) to Fyn were from Santa Cruz Biotechnology and BD Transduction Laboratories, respectively. Ab to p85 α and mAb 4G10 to phosphotyrosine conjugated with peroxidase (HRP) were from Upstate Laboratories. mAb to p85 α was from US Biological. mAb GTU-88 and Abs (Cat. Nos. T5192 and T3195) to γ -tubulin, mAb TUB 2.1 to β -tubulin and TUB 2.1-FITC were from Sigma. Ab to GFP was from Clontech. mAbs TU-31 to γ -tubulin, TU-01 to α -tubulin, and NF-09 to neurofilament protein were specified previously [16]. mAb TU-30 to γ -tubulin conjugated with DY547, mAb Fyn-01 to Fyn and Ab for precipitation of GFP were from Exbio Praha. mAb NF-09 and Ab to nonmuscle myosin (Biomedical Technologies) served as negative controls in precipitation experiments. Abs to catalytic active (pY418) and inactive (pY530) forms of Src, recognizing homologous phosphorylation sites in Fyn, were from BioSource International. Ab to GST was provided by Dr. Pe. Dráber and mAb 20H5 to centrin by Dr. J. L. Salisbury

[21]. Secondary HRP-conjugated Abs were from Promega. FITC-conjugated anti-mouse and indocarbocyanate (Cy3)-conjugated anti-rabbit Abs were from Jackson Immunoresearch Laboratories.

Rabbit Ab to GCP2 was prepared according to previously described procedure [22] and immunoglobulins were affinity-purified on GST-GCP2 protein coupled to CNBr-activated Sepharose. Ab was eluted by 3.5 M MgCl₂ in 5% (v/v) dioxane and dialyzed against PBS, pH 7.5. The Ab specificity was verified by immunoblotting and immunofluorescence microscopy.

Cells

Mouse embryonal carcinoma P19 cells were cultured and subsequently differentiated by 1 μ M RA for 9 days as described previously [16]. In some cases, cells before extraction were incubated for 60 min at 37°C with SU6656, PP2 and PP3 or with imatinib mesylate, an inhibitor of tyrosine kinases Bcr-Abl and c-kit, at concentrations 20 μ M. Alternatively, cells were incubated for 60 min at 37°C with 200 nM wortmannin, an inhibitor of PI3K, or with 10 mM methyl- β -cyclodextrin for depletion of cholesterol. For immunofluorescence visualization on differentiated cells, a modified culturing procedure in the presence of cytosine β -D-arabinofuranoside was applied [16]. HEK293-FT cells (Promega) were transfected with 2-20 μ g DNA per 6-cm tissue culture dish using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested for experiment 48 h after transfection.

Preparation of cell extracts

All cell extracts were prepared at 4°C. For differential centrifugation, cells were rinsed with HEPES buffer (50 mM HEPES pH 7.6, 75 mM NaCl, 1 mM MgCl₂, 1 mM EGTA) and scraped into HEPES buffer supplemented with protease and phosphatase (1 mM Na₃V0₄, 1 mM NaF) inhibitors. Cells were disrupted by sonication (2 x 10 sec pulses, amplitude 20) using 500W Ultrasonic Homogenizer (Cole Parmer), and cell nuclei were removed by centrifugation at 1000 *g* for 10 min while post-nuclear supernatants were centrifuged at 20000 *g* for 15 min. Supernatants (S₂₀) were centrifuged at 55000 *g* for 30 min. Supernatants (S₅₅) were subsequently centrifuged at 100000 *g* for 1 h to obtain high-speed supernatants (S₁₀₀) and high-speed pellets (P₁₀₀). Alternatively, supernatants (S₂₀) were supplemented with buffer or with NP-40 to final concentration 0.5% (v/v), incubated for 30 min and centrifuged at 100000 *g* for 1 h. Supernatants without (S) or with NP-40 (SN) were mixed with 5x concentrated SDS-sample buffer, whereas the corresponding pellets (P, PN) were resuspended in 1x SDS-sample buffer in a volume which equalled that of the supernatants. In some experiments, pellets (P₁₀₀) were resuspended with HEPES buffer supplemented with one of the following detergents: Brij 98, NP-40 or Triton X-100 at concentration 1% (v/v). After 30 min of incubation the detergent-insoluble material was centrifuged at 55000 *g* for 30 min.

Cell extracts for GST pull-down assays [1% (v/v) NP-40 in HEPES buffer] were prepared as described [16]. For precipitation from cytosolic fraction, supernatant (S₁₀₀) was adjusted with NP-40 to 1% (v/v) final concentration. For precipitation from microsomal fraction (P₁₀₀), hereafter referred to as membranes, the pelleted material was resuspended in HEPES buffer supplemented with 1% (v/v) NP-40. A volume equal to the corresponding supernatant was used for resuspension of the pellet. Suspension was homogenized by sonication (5 sec pulse, amplitude 10) and after 15 min incubation spun down at 20000 *g* for 15 min.

Density gradient centrifugation

For density gradient centrifugation, cells were extracted with ice-cold 1% (v/v) Triton X-100 in HEPES buffer supplemented with protease and phosphatase inhibitors for 10 min at 4°C and centrifuged at 20000 *g* for 15 min at 4°C. Where indicated, 0.2% (w/v) saponin was added to extraction buffer. 10-40% (w/v) step-density gradients in HEPES buffer were prepared in Ultra-Clear tubes (14 x 95 mm, Beckman). Extracts were adjusted to 40% (w/v) sucrose by adding equal volumes of 80% (w/v) sucrose in HEPES buffer. 3 ml were loaded into a tube, followed by 7 ml of 30% (w/v) sucrose and 2 ml of 10% (w/v) sucrose. Gradients were centrifuged at 35000 rev./min in Beckman SW40 rotor for 21 h at 4°C, and fractionated from the top into eleven fractions. The floating detergent-resistant membranes (DRM) were concentrated in fraction No. 2. To concentrate DRM, fraction No. 2 was diluted with 6 volumes of ice-cold water and centrifuged at 35000 rev./min in Beckman SW40 rotor for 1 h at 4°C. For SDS-PAGE pelleted DRM were mixed with SDS-sample buffer corresponding to 1/30 volume of the No. 2 fraction. Alternatively, pelleted DRM were resuspended in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM MgCl₂, 1 mM EGTA) and used for microtubule spin-down assay.

Immunoprecipitation, kinase assay and immunoblotting

Immunoprecipitation was performed as described [16]. Beads of protein A were saturated with: (I) Ab to Fyn, (II) Ab to p85 α , (III) Ab to GFP, (IV) negative control Ab to non-muscle myosin, (V) mAb TU-31 (IgG2b) against γ -tubulin, (VI) negative control mAb NF-09 (IgG2a), or (VII) immobilized protein A alone. For comparison of immunoprecipitation from unstimulated and stimulated cells, extracts were adjusted to the same protein concentration. Alternatively, beads with immunoprecipitated material were used for *in vitro* kinase assay as described [7]. The ³²P labelled-immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and the ³²P-labelled proteins were detected by bioimaging analyzer BAS-5000 (Fuji Photo Film).

Details of immunoblotting are described elsewhere [23]. Anti-tubulin mAbs TU-01 (spent culture supernatant), TUB 2.1 and GTU-88 were diluted 1:50, 1:2000 and 1:5000, respectively. mAbs to Fyn, p85 α , centrin and phosphotyrosine were diluted 1:300, 1:500, 1:1000 and 1:30000, respectively. Abs to GFP and GCP2 were diluted 1:500, while Ab to GST was diluted 1:30000. Abs to pY418 and pY530 were diluted 1:5000. Secondary HRP-conjugated Abs were diluted 1:10000. Bound Abs were detected by chemiluminescence reagents.

GST pull-down assay

GST pull-down assays with cell extracts were performed as described [16]. Alternatively, sedimented beads with immobilized GST- γ -tubulin were incubated with PI3K at concentration 0.4 μ g/ml in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl). After washing, bound proteins were eluted by boiling in SDS-sample buffer. In some experiments, 0.1 μ g of active Fyn was added to beads with immobilized GST fusion proteins and subjected to kinase assay.

Immune complex PI3K assay

PI3K activity was determined essentially as described [24]. After immunoprecipitation, the beads were washed in kinase buffer and suspended in kinase buffer with or without 100 nM wortmannin. After 10 min incubation at 25°C, the assay was initiated by addition of kinase

buffer containing sonicated phosphatidylinositol and [γ - 32 P]ATP, and the reaction mixture was incubated for 30 min at 25°C.

Microtubule spin-down assay and immunofluorescence

Membranes were washed in BRB80 buffer and resuspended in BRB80 by sonication (5 sec pulse, amplitude 10). Spin-down assay was performed as described [25]. Membranes were incubated with 10 μ M tubulin in the presence of 1 mM GTP for 20-30 min at 37°C. After fixation with 1% (v/v) glutaraldehyde, membranes were centrifuged through 10% (v/v) glycerol cushion on glass coverslips, postfixed in cold methanol and used for immunostaining. Alternatively, concentrated DRM were used for the assay. In salt-washed experiments the membranes were first incubated in 2 M KCl for 1h on ice, then pelleted, extensively washed in BRB80 at 4°C and finally resuspended in BRB80 by sonication. In recovery experiments, salt-washed membranes were incubated for 1 h at room temperature with cytosol (S₁₀₀) preabsorbed with negative control Ab or with cytosol immunodepleted of γ -tubulin. After washing in BRB80, they were used for the assay. Immunodepletion of γ -tubulin was performed by incubation of 600 μ l cytosol, diluted 1:5 in HEPES buffer, with protein A beads saturated with a mixture of polyclonal Abs to γ -tubulin. After 45 min incubation at room temperature, the beads were pelleted and supernatant was subjected to additional three subsequent absorption steps with new Ab-saturated beads. The same procedure was performed with negative control Ab.

Preparations after spin-down assay were stained for tubulin with TUB 2.1-FITC (1:50). TUB 2.1-FITC and anti- γ -tubulin mAb TU-30 conjugated with DY547 (1:500) were used for double-label staining. Alternatively, samples were stained with TUB 2.1-FITC and anti-Fyn (1:500) or anti-p85 α (1:2000) Abs, followed by Cy3-conjugated anti-rabbit Ab (1:500).

Immunofluorescence microscopy on fixed cells was performed as described [23]. Cells were fixed in 3% (w/v) formaldehyde and extracted thereafter with 0.5% (v/v) Triton X-100. The samples were double-label stained with rabbit Ab to γ -tubulin (1:500) and mAb Fyn-01 (1:100) to Fyn, followed by Cy3-conjugated anti-rabbit Ab and FITC-conjugated anti-mouse Ab (1:200). The preparations were examined with Olympus A70 Provis microscope. Conjugates alone did not give any detectable staining.

RESULTS

Membrane-associated γ -tubulin in differentiated P19 cells

First, we determined the distribution of γ -tubulin in RA-differentiated P19 cells. Immunoblot analysis of fractions after differential centrifugation of cell homogenate revealed that γ -tubulin associated with both the pelleted materials (P₂₀, P₅₅, P₁₀₀) and the supernatants (S₂₀, S₅₅, S₁₀₀). On the other hand centrin, a marker of centrosomes, localized exclusively in low-speed pellet (P₂₀) (Figure 1A).

To determine the relative distribution of tubulins and kinases, supernatants (S₂₀) were supplemented or not with 0.5% (v/v) NP-40 before centrifugation at 100000 *g* for 60 min. In the absence of detergent γ -tubulin was equally distributed between cytosolic (S₁₀₀) and membrane (P₁₀₀) fractions. In contrast, β -tubulin and p85 α predominated in cytosolic fraction, while Fyn prevailed mainly in membrane fraction (Figure 1B). Distribution of α -tubulin resembled that of β -tubulin (not shown). γ -Tubulin associated with the detergent-resistant fraction (PN₁₀₀) even in the presence of 0.5 % (v/v) NP-40. Under such conditions β -tubulin prevailed mainly in detergent-soluble fraction (SN₁₀₀). Distributions of Fyn and p85 α in SN₁₀₀

and PN₁₀₀ fractions resembled that of γ -tubulin (Figure 1C). When membrane-associated γ -tubulin (P₁₀₀) was extracted with lysis buffer containing 1% (v/v) Brij 98, 1% (v/v) NP-40 or 1% (v/v) Triton X-100, approximately 50%, 45% and 40%, respectively, of γ -tubulin was still associated with detergent-resistant material. These data indicate that the distribution patterns of γ -tubulin and $\alpha\beta$ -tubulin dimers differ, and that a substantial part of γ -tubulin is present in cellular fraction containing Fyn. A part of γ -tubulin associated with microsomal fraction is resistant to treatment with non-ionic detergents.

Double-label immunostaining of differentiated cells with anti- γ -tubulin and anti-Fyn Abs revealed that both proteins were located in rounded cell bodies and in neurite projections. Colocalization of Fyn and γ -tubulin was observed in some regions of thicker processes (see Supplementary Figure 1S).

Nucleation of microtubules from membranes is dependent on γ -tubulin and kinases

In vitro microtubule assembly assay was performed to test whether or not the membrane-associated γ -tubulin is capable of nucleating microtubules. Membranes were incubated for 20 or 30 min in a buffer supplemented with 10 μ M $\alpha\beta$ -tubulin dimer and 1 mM GTP. The spin-down assay revealed that microtubules grew from membranes (Figure 2A, a, c, e) that contained γ -tubulin (Figure 2A, b), Fyn (Figure 2A, d) as well as p85 α (Figure 2A, f). On the other hand, centrin was not detected on membranes, suggesting that nucleation is not attributable to remnants of centrosomes. The growth of microtubules was time-dependent. We have noted that all membrane fragments stained for β -tubulin after incubation with tubulin dimers, similarly as it was reported for isolated Golgi membranes [4], but only some nucleated microtubules. To determine whether γ -tubulin is responsible for microtubule nucleation from membranes, isolated membranes were treated with 2M KCl. Under these conditions nucleation was not detected (Figure 2B, a). In recovery experiment, salt-treated membranes were incubated with cytosol preabsorbed with negative control Ab prior to *in vitro* microtubule assembly assay. In this case, nucleation of microtubules was restored (Figure 2B, b). When salt-treated membranes were incubated with cytosol immunodepleted of γ -tubulin, nucleation of microtubules was absent (Figure 2B, c). No spontaneous microtubule assembling took place when $\alpha\beta$ -tubulin dimer was incubated in the absence of membranes (Figure 2B, d). Immunoblotting experiments confirmed a substantially higher amount of γ -tubulin in untreated membranes when compared to salt-washed membranes. γ -Tubulin reoccurred after incubation of salt-washed membranes with cytosol preabsorbed with negative control Ab. However, when salt-treated membranes were incubated with cytosol immunodepleted of γ -tubulin, no increase in γ -tubulin was observed. The amount of Fyn on membranes did not substantially change after salt-treatment (see Supplementary Figure 2S).

When cells were pretreated with the Src family kinases inhibitors SU6556 or PP2 at 20 μ M, microtubule nucleation was inhibited (Figure 2C, b). Nucleation was not affected when DMSO alone (Figure 2C, a) was used. It is known that PP2 also inhibits tyrosine kinases Kit and Bcr-Abl, in addition to Src family kinases [26]. Nucleation experiments were therefore performed with membranes isolated from cells pretreated with 20 μ M imatinib mesylate inhibiting kinases Kit and Bcr-Abl. In this case no inhibition was observed (not shown). Pretreatment of cells with 200 nM wortmannin also inhibited microtubule nucleation on isolated membranes (see Supplementary Figure 3S, A). Pretreatment with wortmannin or PP2 did not affect association of γ -tubulin with membranes (see Supplementary Figure 3S, B). These *in vitro* experiments suggest that γ -tubulin associated with microsomal fraction has the capability to nucleate microtubules, and that the process depends on activity of Src family kinases and PI3K.

Membrane-associated γ -tubulin interacts with Fyn and p85 α

A series of precipitation experiments was performed to find out whether γ -tubulin and Fyn form complexes in membrane fractions of differentiated P19 cells. Anti-Fyn Ab coprecipitated γ -tubulin as well as p85 α (Figure 3A). After precipitation with a negative control Ab, no specific staining was detected with Abs to Fyn, γ -tubulin and p85 α . As Fyn formed complexes with p85 α , we intended to find out whether p85 α could serve in P19 cells as a substrate for Fyn. *In vitro* kinase assay revealed protein phosphorylation in Fyn membrane immunocomplexes isolated from both resting and differentiated cells. A protein corresponding to the p85 α , as confirmed by immunoblotting with specific Ab, showed enhanced phosphorylation in differentiated cells (Figure 3B, 0 and 9). This phosphorylation was inhibited by pretreatment of the cells with PP2 (Figure 3B, + PP2). On the other hand treatment with PP3 was without effect (not shown). No kinase activity was detected after precipitation with the negative control Ab (Figure 3B, c).

Complexes of γ -tubulin with p85 α have been previously described in cells overexpressing insulin receptor [13,14]. Next we therefore examined the possibility that membrane-bound γ -tubulin associates with p85 α in differentiated P19 cells. Ab to p85 α coprecipitated γ -tubulin and its amount was higher in immunocomplexes from membrane fraction (Figure 3C). To corroborate the results of immunoblotting experiments, PI3K activity was also evaluated by immunocomplex kinase assay. PI3K activity associated with membrane-bound γ -tubulin was found in both resting and differentiated cells. However, a 1.82 ± 0.24 (mean \pm S.D., $n=3$) fold increase in PI3K activity was detected in differentiated cells. Pretreatment of immunocomplexes with 100 nM wortmannin completely inhibited the formation of ^{32}P -labelled PIP (Figure 3D). Summarily these data demonstrate that membrane bound γ -tubulin in differentiated P19 cells forms complexes with both Fyn and active PI3K.

γ -Tubulin associates with detergent-resistant membranes

To determine whether γ -tubulin is also associated with DRM, cells were solubilized in 1% (v/v) Triton X-100 and lysates were fractionated by equilibrium density gradient centrifugation. To make the results comparable, the same protein amounts were loaded for centrifugation and the corresponding blots were exposed on the same autoradiography film. Fyn served as marker of DRM. Immunoblotting experiments revealed substantial differences in the distribution of γ -tubulin in the course of RA-induced differentiation. In undifferentiated cells (day 0) both Fyn and γ -tubulin were only found in high-density (HD) fractions, while in differentiated cells (day 9) they were detected in both HD and low-density (LD) fractions (Figure 4A). LD fractions correspond to floating DRM while HD fractions correspond to fractions not associated with DRM. Since DRM are rich in cholesterol, differentiated cells were extracted with buffer containing 1% (v/v) Triton X-100 and 0.2% (v/v) saponin, which is known to sequester cholesterol. Both Fyn and γ -tubulin were absent in LD fractions in saponin-treated samples (Figure 4A, Sap). Comparable effect was observed when cholesterol was depleted by preincubation of cells with methyl- β -cyclodextrin.

Complementary immunoblotting experiments were performed to compare the relative distribution of γ -tubulin and Fyn in total cell extracts and DRM. The amounts of proteins in whole-cell extract (lane 1 in Figure 4B) corresponded to those in concentrated DRM (lane 2 in Figure 4B). γ -Tubulin was present in DRM in a relatively high content in comparison with β -tubulin. Importantly, the GCP2 protein, a component of γ -tubulin complexes, was detected in DRM. As expected, Fyn was highly enriched with DRM. The microtubule nucleation

capacity of concentrated DRM was demonstrated by spin-down assay. Microtubules grew from DRM (Figure 4C, a) that contained γ -tubulin (Figure 4C, b). Immunoprecipitation experiments with anti-Fyn Ab followed by immunoblotting with Ab to γ -tubulin confirmed an association of γ -tubulin with Fyn in DRM (not shown). Collectively, these data indicate that a subpopulation of γ -tubulin is associated with DRM and forms there complexes with Fyn.

Association of Fyn with membrane is important for its interaction with γ -tubulin

To answer the question whether association of Fyn with membranes is important for its interaction with γ -tubulin, EGFP-tagged Fyn construct was prepared. The EGFP tag was added to the C-terminus of Fyn, and the distribution of Fyn-EGFP was determined in soluble and membrane fractions of transfected undifferentiated P19 cells. Overexpressed Fyn-EGFP was present in both soluble and membrane fractions, though a higher amount was associated with the latter. On the other hand EGFP, that served as control, was found exclusively in soluble fraction (Figure 5A, Load). Anti- γ -tubulin Ab precipitated γ -tubulin from both fractions, yet coprecipitated only membrane targeted Fyn-EGFP, but not cytosolic Fyn-EGFP or EGFP (Figure 5A, IP). Anti-phosphotyrosine Ab stained neither soluble nor membrane-bound γ -tubulin in immunoprecipitates (not shown). This suggests that γ -tubulin is not phosphorylated by Fyn. To rule out the possibility that cytosolic Fyn-EGFP is not folded properly and therefore not accessible to interaction with γ -tubulin, we have used *in vitro* kinase assay as a measure of proper folding. Membrane and cytosolic Fyn-EGFP were precipitated by Ab to GFP, and the immunoprecipitated material was used for *in vitro* kinase assay. Both membrane and cytosolic Fyn-EGFP precipitates were phosphorylated and contained an active form of Fyn as documented by autoradiography and by immunoblotting with Ab to the autophosphorylation site pY418 (Figure 5B). While Ab to the negative regulatory site pY530 also reacted with membrane and cytosolic form of Fyn-EGFP, non-active Fyn-EGFP was not accumulated in cytosol (not shown). Combined data suggest that both membrane and cytosolic Fyn-EGFP contains a mixture of active and non-active forms of kinase. As γ -tubulin did not interact with Fyn-EGFP in cytosol, these two proteins apparently associate indirectly via adaptor protein(s) located in membranes.

γ -Tubulin binds directly to the C-terminal SH2 domain of p85 α , outside the tyrosine-phosphorylated protein-recognition site

Because γ -tubulin associated with p85 α in differentiated P19 cells, we attempted to identify the region in p85 α molecule responsible for interaction with γ -tubulin. The pull-down assays with GST-tagged p85 α domains (Figure 6A) and extracts from differentiated cells revealed that γ -tubulin associated with all the fusion proteins containing the C-terminal SH2 (cSH2) domain. No interaction was observed with fusion proteins containing SH3-RhoGAP fragment, the N-terminal SH2 (nSH2) domain or GST alone (Figure 6B, γ -Tb). α -Tubulin interacted with the same fragments, except for the cSH2 domain (Figure 6B, α -Tb). The amounts of immobilized GST fusion proteins were similar, as evidenced by staining with anti-GST Ab (not shown). These results indicate that association of γ -tubulin with the cSH2 domain is independent of the presence of $\alpha\beta$ -tubulin dimer. All SH2 domains contain a conserved F-L-V-R motif, which is essential for the phosphotyrosine binding. Mutation of the critical arginine residue in the F-L-V-R motif abolishes the capability of SH2 domain to bind phosphotyrosine proteins [27]. When the pull-down assay was performed with fusion protein containing mutated arginine in cSH2 domain (GST-cSH2-R649L), the binding of γ -tubulin to this fusion protein was preserved (Figure 6B, γ -Tb). To decide whether γ -tubulin interacts

with p85 α directly, the GST pull-down assay was performed with GST- γ -tubulin and recombinant PI3K (p110 δ complexed to p85 α). Though PI3K did bind to GST- γ -tubulin, it failed to bind to GST fusion protein containing fragment of GCP2 (negative control) or GST alone (Figure 6C, p85 α). The amounts of immobilized GST fusion proteins were similar (Figure 6C, GST).

To prove that the cSH2 domain is responsible for γ -tubulin binding *in vivo*, p85 α and its truncated forms (Figure 7A) were transfected into HEK cells. All constructs were expressed in the cells (Figure 7B, Load, p85 α) and did not alter the expression of γ -tubulin (Figure 7B, Load, γ -Tb). Cell extracts were precipitated with Ab to γ -tubulin and blots were probed with Ab to p85 α . As expected, the amount of p85 α in complex with γ -tubulin increased after overexpression of p85 α . However, the truncated forms p85- Δ CT and p85-inter Δ CT without cSH2 domain were absent in γ -tubulin immunocomplexes. In contrast, p85 α with deleted RhoGAP domain, but with intact cSH2 domain, was present there (Figure 7B, IP, p85 α). When the C-terminal fragment of p85 α containing cSH2 domain (CT) was expressed in cells, a dose-dependent decrease of p85 α in γ -tubulin immunocomplexes was evident (Figure 7C, IP, p85 α), while the expression level of p85 α and γ -tubulin was unchanged (Figure 7C, Load). Collectively these data suggest that γ -tubulin directly interacts with the cSH2 domain and that this interaction is outside the tyrosine-phosphorylated protein-recognition site on the cSH2 domain of p85 α .

DISCUSSION

It is known that the bulk of cytoplasmic γ -tubulin is not associated with MTOCs [28]. Here we show that γ -tubulin in RA-differentiated P19 cells is equally distributed to both soluble and membrane fractions. A fraction of membrane-associated γ -tubulin was resistant to non-ionic detergents. On the other hand, $\alpha\beta$ -tubulin dimers prevailed in soluble pool. Interestingly, γ -tubulin in differentiated cells was found not only in rough membrane fractions but also in DRM. Since γ -tubulin itself does not have a consensus membrane-binding motif, it is likely that other membrane-anchored proteins interacting with γ -tubulin mediate this association. In differentiated cells only a small fraction of $\alpha\beta$ -tubulin dimers is present in DRM, contrary to γ -tubulin. Therefore it seems unlikely that tubulin dimers dock γ -tubulin to membranes. It is more probable that Src family kinases and/or adapter proteins accumulating in DRM [29] take part in this docking.

Microtubules are essential for neurite formation, and Fyn kinase plays a key role in this process. We have previously demonstrated an enhanced interaction of γ -tubulin with Fyn in RA-differentiated cells [16]. Several lines of new evidence indicate that this interaction occurs predominantly in membrane fraction. First, Fyn was associated with γ -tubulin in immunoprecipitates from membranes. Second, complexes of both proteins were present in DRM that are known to accumulate Fyn. Third, γ -tubulin and Fyn were located on membranes from which microtubules were nucleated. Fourth, the nucleation activity of membrane-associated γ -tubulin was inhibited by pretreatment of cells with Src-family specific inhibitors; this implies that Src family kinases might play a role in modulation of microtubule nucleation from membranes. Finally, tests with EGFP-tagged Fyn showed that association of Fyn with membranes was required for interaction with γ -tubulin. Although this study was focused on Fyn, one cannot rule out that other members of the Src family could also modulate microtubule nucleation.

In differentiating cells, the enhanced interaction of γ -tubulin with Fyn was accompanied by increased activity of PI3K associated with γ -tubulin. Immunoprecipitation experiments

confirmed the association of γ -tubulin with p85 α . Although *in vitro* interactions between the γ -tubulin and p85 α have been reported [13,14], no conclusions were drawn on the exact location of γ -tubulin binding site on p85 α molecule and the type of interaction between these two proteins. Our data from GST pull-down assays with p85 α domains demonstrate that γ -tubulin binds to cSH2 domain and that phosphotyrosine-mediated binding is not required for this interaction. Moreover, GST pull-down assay with γ -tubulin fusion protein and recombinant PI3K confirms a direct interaction. Immunoprecipitation experiments from transfected cells, expressing truncated forms of p85 α , or overexpressing cSH2 domain, demonstrate that the cSH2 domain is essential for interaction of p85 α with γ -tubulin *in vivo*. The capability of the cSH2 domain of p85 α to mediate phosphorylation-independent interaction has been reported for serine/threonine kinase A-Raf [30], and the same may hold true for γ -tubulin.

It has been shown that the cSH2 domain of p85 α regulates the activity of PI3K. This domain carries the Y688 that can be phosphorylated by cytoplasmic kinases [10]. Phosphorylation of this site then functionally blocks the inhibitory effect of p85 α on the catalytic subunit. Neutralization of the inhibitory domain renders p85 α responsive to PI3K activation signals that are transduced by small GTPases [10]. Interestingly, reduced PI3K activity was reported in activated mast cells isolated from Fyn knock-out mice [31]. Here we show that Fyn in P19 cells can phosphorylate p85 α *in vivo* and that phosphorylation is enhanced in differentiated cells. The efficacy of Fyn in phosphorylating p85 α was further confirmed by *in vitro* kinase assay with GST-cSH2 and purified active Fyn (see Supplementary Figure 4S). This indicates that Fyn in P19 cells phosphorylates the cSH2 of p85 α and thus regulate the activity of catalytic subunit.

The results obtained by microtubule spin-down assay show that membrane-associated γ -tubulin complexes can nucleate microtubules from both rough membrane fractions and DRM. In this connection it should be noted that γ -tubulin associates with vesicular structures and Golgi-derived vesicles [4,5] and it is essential for microtubule nucleation from Golgi membranes [4,32]. Although DRM are most abundant in plasma membrane, they first assemble in the Golgi apparatus [33]. It is therefore uncertain whether microtubule formation from DRM reflects nucleation from the plasma membrane or other membranous components.

Our data indicate that other microtubule nucleating sites associated with membrane-associated γ -tubulin may exist in addition to centrosomes in differentiating P19 cells. The process of microtubule nucleation on membranes could be regulated by Src family kinases and PI3K. This notion is supported by our data indicating that upregulation of Fyn leads to Fyn accumulation in the membranes and phosphorylation of p85 α . It could lead to activation of p110 catalytic subunit and initiation of PI3K signaling pathway. Recruitment of p85 α with bound γ -tubulin into membranes, probably through an adaptor protein, might contribute to membrane-associated microtubule nucleation. Since pretreatment of cells with wortmannin also inhibited microtubule nucleation on isolated membranes, PI3K itself, or some other proteins in the PI3K signaling pathway, could participate in the modulation of microtubule nucleation.

In conclusion, the presented data show that γ -tubulin in differentiating P19 cells is associated with membranes including DRM. Membrane-associated γ -tubulin is involved in nucleation of microtubules, and Src family kinases and PI3K play an important role in this process. Membrane-associated γ -tubulin forms complexes with Fyn and directly interacts with cSH2 domain of p85 α . The findings suggest that proteins of the signaling cascades could modulate non-centrosomal microtubule nucleation by membrane-associated γ -tubulin.

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FIGURE LEGENDS

Figure 1 Distribution of γ -tubulin and kinases in RA-differentiated P19 cells

(A) Relative distribution of γ -tubulin (γ -Tb) and centrin (Cen) in fractions after differential centrifugation of cell extract. Cell fractions were prepared as described in Material and Methods. (B) Immunoblotting of supernatants (S₁₀₀) and pellets (P₁₀₀) with Abs to γ -tubulin (γ -Tb), β -tubulin (β -Tb), Fyn and p85 α . (C) Immunoblotting of supernatants (SN₁₀₀) and pellets (PN₁₀₀) after centrifugation in the presence of 0.5% (v/v) NP-40. To compare the relative distribution of proteins, pelleted material was resuspended in a volume equal to that of the corresponding supernatant.

Figure 2 Assembly of microtubules from membranes

Isolated membranes were incubated for 20 min at 37°C with 10 μ M tubulin and 1 mM GTP. After fixation, samples were centrifuged onto coverslips and used for immunostaining. (A) Double-label staining of membranes with assembled microtubules. Staining of β -tubulin (a,c, e), γ -tubulin (b), Fyn (d) and p85 α (f). (B) Staining of salt-washed membranes (a), salt-washed membranes incubated with cytosol (b) and salt-washed membranes incubated with cytosol immunodepleted of γ -tubulin (c). Negative control without membranes (d). Staining of β -tubulin (a-d). (C) Membranes from control cells (a) or cells pretreated with PP2 (b) were incubated with tubulin and after spin-down stained for microtubules. Bar, 10 μ m in A-C.

Figure 3 Coimmunoprecipitation of membrane-bound γ -tubulin with Fyn and p85 α

(A) Soluble (S) and membrane (P) fractions from differentiated cells were precipitated with Ab to Fyn and blots were probed with Abs to Fyn, γ -tubulin (γ -Tb) and p85 α . Immobilized Ab not incubated with cell extract (C, control). White arrowheads point to heavy chain of IgG. (B) Kinase activity in Fyn membrane immunocomplexes isolated from resting (0) and differentiated (9) cells pretreated with (+) or without (-) PP2 inhibitor. Kinase activity after precipitation with negative control Ab (C). Precipitated Fyn was detected by immunoblotting. Positions of p85 α and molecular mass marker (in kDa) are indicated on the left. (C) Soluble (S) and membrane (P) fractions from differentiated cells were precipitated with Ab to p85 α and blots were probed with Abs against p85 α and γ -tubulin (γ -Tb). Immobilized Ab not incubated with cell extract (C, control). White arrowhead points to heavy chain of IgG. (D) Activity of PI3K in γ -tubulin membrane immunocomplexes isolated from resting (0) and differentiated (9) cells. In labelled samples, the immunocomplexes were exposed to wortmannin (w). Precipitated γ -tubulin was detected by immunoblotting (γ -Tb). Migration of phosphatidylinositol phosphate (PIP) and starting point of thin layer chromatography (S) are denoted.

Figure 4 Distribution of Fyn and γ -tubulin during density gradient centrifugation of lysates from resting and RA-differentiated P19 cells

Cells were extracted in lysis buffer with 1% (v/v) Triton X-100 in the absence or presence of cholesterol-depleting agent saponin (Sap), and subjected to equilibrium density gradient centrifugation. Fractions were separated by SDS-PAGE and immunoblotted for the corresponding proteins. (A) Staining with Abs to Fyn and γ -tubulin (γ -Tb). Numbers 0 and 9 indicate days of incubation cells with RA. Numbers at the bottom indicate individual fractions (fr.). LD and HD denote low-density and high-density fractions, respectively. (B) Relative

distribution of proteins in whole-cell extracts (lane 1) and concentrated DRM (lane 2). Staining with Abs to γ -tubulin (γ -Tb), β -tubulin (β -Tb), GCP2 and Fyn. The same amount of proteins (0.3 μ g) was loaded into each lane. (C) Assembly of microtubules from DRM. Concentrated DRM were incubated with tubulin and after spin-down double-label stained for β -tubulin (TUB) and γ -tubulin (γ -Tb). Bar, 10 μ m.

Figure 5 Association of γ -tubulin with Fyn in cells expressing EGFP-tagged Fyn

(A) Soluble (S) and membrane (P) fractions from cell expressing Fyn-EGFP (a) and EGFP (b) proteins were precipitated with Ab to γ -tubulin and blots from extracts (Load) and precipitates (IP) were probed with Abs to GFP, Fyn and γ -tubulin (γ -Tb). (B) Soluble (S) and membrane (P) fractions from cell expressing EGFP and Fyn-EGFP proteins were precipitated with Ab to GFP. Kinase activity in GFP immunocomplexes (32 P), immunoblots with Ab to catalytic active form of Fyn (pY418) and with Ab to GFP. To compare the relative distribution of proteins, pelleted material was resuspended in a volume equal to that of the corresponding supernatant.

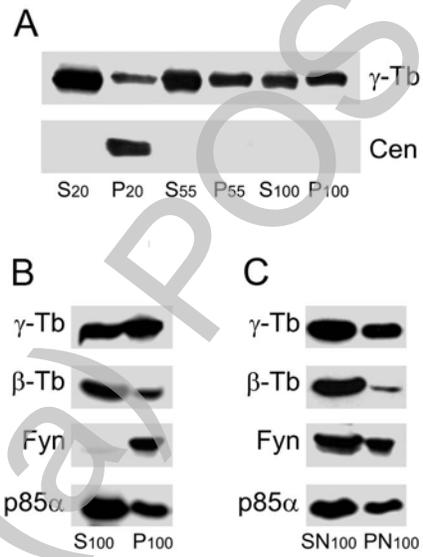
Figure 6 Interaction of γ -tubulin with p85 α domains in GST pull-down assay

(A) Schematic diagram of GST-p85 α (a) and its truncated forms (b-e) used in pull-down assays. (B) Lysates from differentiated cells (Load) were incubated with GST-fusion proteins or GST alone immobilized on Glutathion Sepharose beads. Blots of bound proteins were probed with Abs to γ -tubulin (γ -Tb) and α -tubulin (α -Tb). (C) Binding of PI3K to GST- γ -tubulin. PI3K (p110 δ complexed to p85 α) was incubated with immobilized GST- γ -tubulin, GST alone and GST-GCP2 fragment. Alternatively, PI3K was incubated with beads without GST-fusion proteins (carrier alone). Blots of bound proteins were probed with Abs to p85 α (p85 α) or GST. Molecular-mass markers (in kDa) are indicated on the left.

Figure 7 Interaction of γ -tubulin with p85 α truncated forms expressed in cells

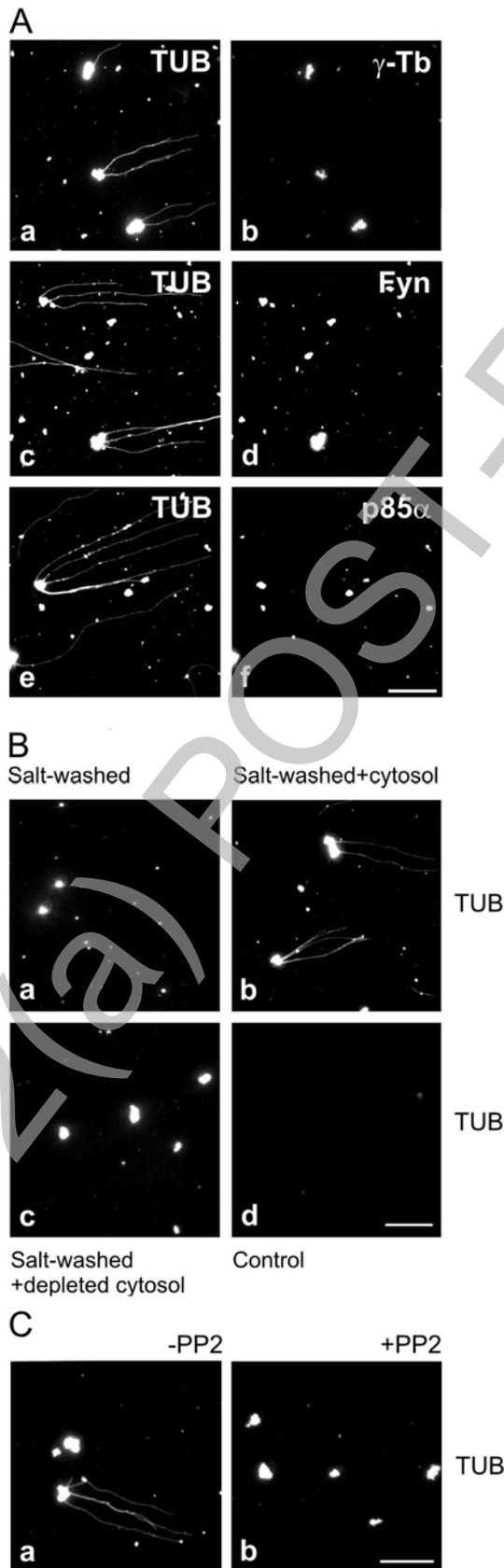
(A) Schematic diagram of p85 α (a) and its truncated forms (b-d) used for expression in HEK cells. (B) Whole-cell extracts (Load) were precipitated with Ab to γ -tubulin (IP) and blots were stained with Abs to p85 α and γ -tubulin (γ -Tb). Wild-type cells (wt), cells expressing exogenous p85 α (a), p85 α - Δ CT (b), p85 α - Δ interCT (c) or p85 α - Δ RhoGAP (d). Molecular-mass markers (in kDa) are indicated on the left. (C) Whole-cell extracts (Load) of HEK cells transfected with increasing amounts of DNA coding the C-terminal region of p85 α (CT) were precipitated with Ab to γ -tubulin (IP). Blots were stained with Abs to p85 α and γ -tubulin (γ -Tb). Wild-type cells (wt) and cells transfected with 5 μ g (lane 1), 10 μ g (lane 2) and 20 μ g (lane 3) of DNA.

Figure 1



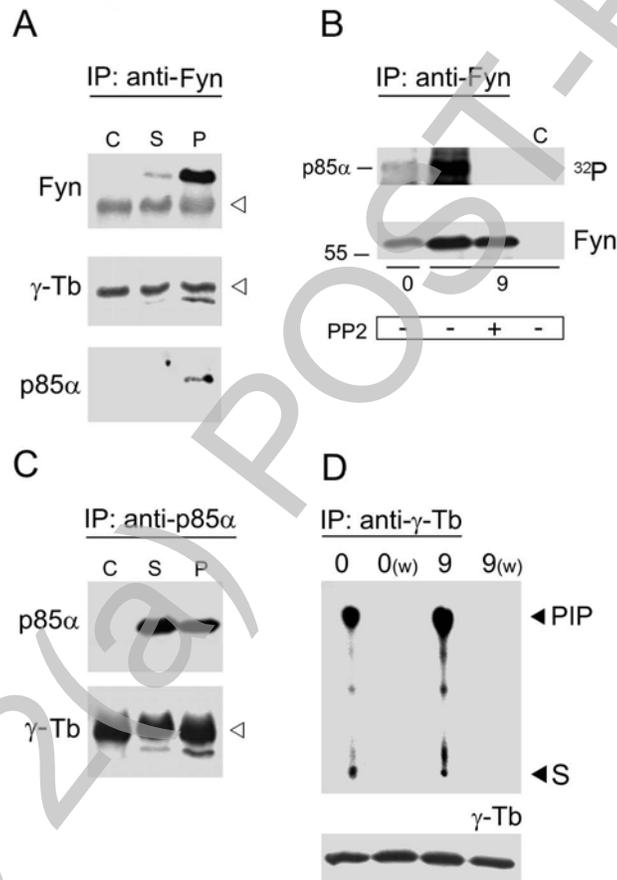
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Figure 2



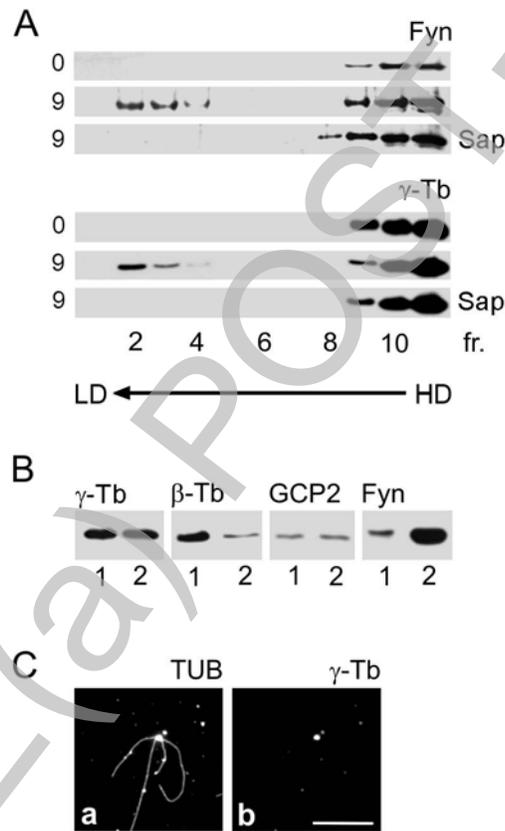
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Figure 3



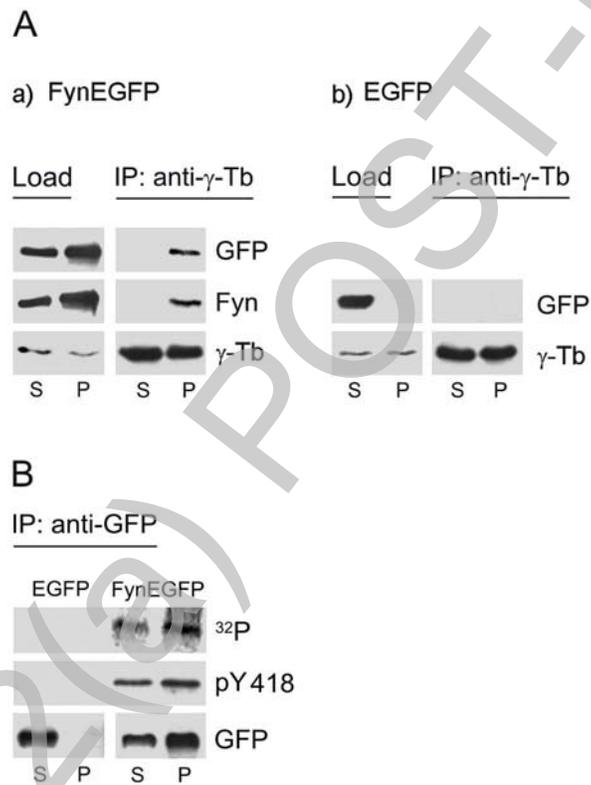
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Figure 4



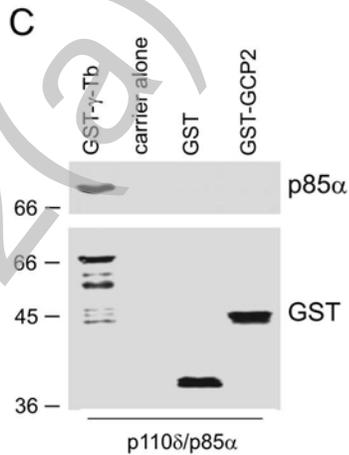
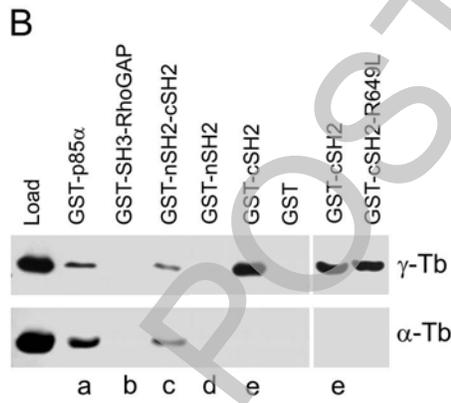
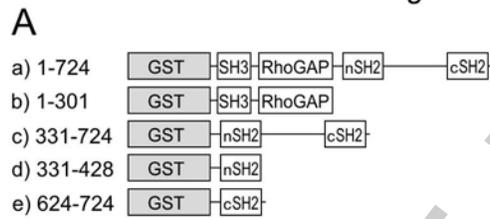
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Figure 5



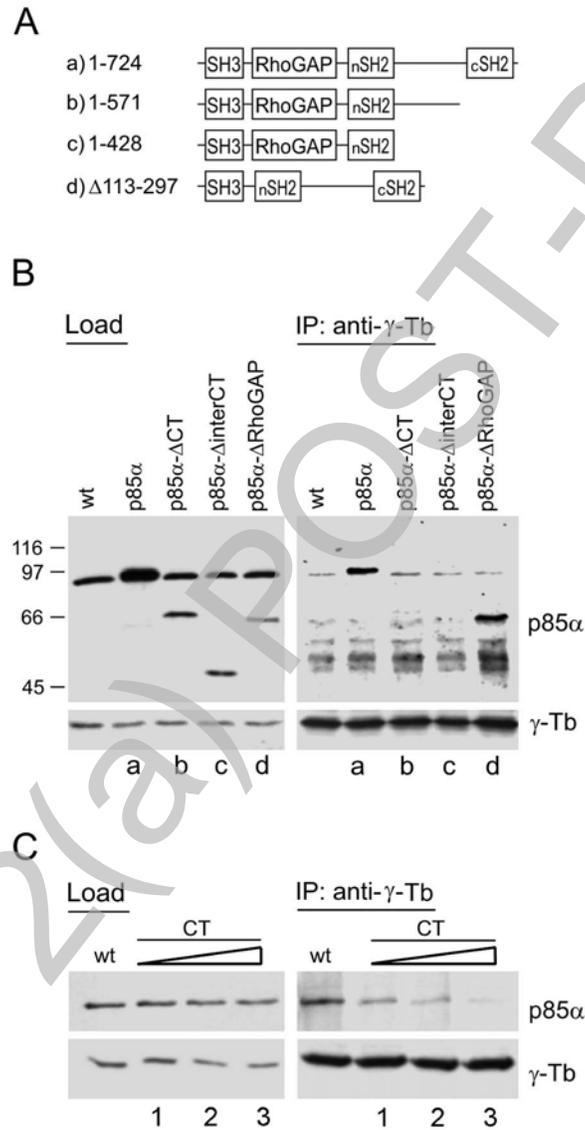
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Figure 6



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Figure 7



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