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

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The 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 results suggested that tyrosine kinases might serve as regulators of γ-tubulin function, their exact roles remain enigmatic. In the present study, we show that a pool of γ-tubulin associates with detergent-resistant membranes in differentiating P19 embryonal carcinoma cells, which exhibit elevated expression of the Src family kinase Fyn (protein tyrosine kinase p59^{Fyn}). Microtubule-assembly assays demonstrated that membrane-associated γ-tubulin complexes are capable of initiating the formation of microtubules. Pretreatment of the cells with Src family kinase inhibitors or wortmannin blocked the nucleation activity of the γ-tubulin complexes. Immunoprecipitation experiments revealed that membrane-associated γ-tubulin forms complexes with Fyn and PI3K (phosphoinositide 3-kinase). Furthermore, *in vitro* kinase assays showed that p85α (regulatory p85α subunit of PI3K) serves as a Fyn substrate. Direct interaction of γ-tubulin with the C-terminal Src homology 2 domain of p85α was determined by pull-down experiments and immunoprecipitation experiments with cells expressing truncated forms of p85α. The combined results suggest that Fyn and PI3K might take part in the modulation of membrane-associated γ-tubulin activities.

Key words: detergent-resistant membrane (DRM), Fyn (protein tyrosine kinase p59^{Fyn}), phosphoinositide 3-kinase (PI3K), PI3K regulatory subunit (p85α), Src homology 2 (SH2) domain, γ-tubulin.

INTRODUCTION

In most proliferating and migrating animal cells, the 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]. γ-Tubulin is associated with other proteins in cytoplasmic complexes. γTuRCs (large γ-tubulin ring complexes) are formed by γTuSCs (γ-tubulin small complexes), comprising two molecules of γ-tubulin and one molecule each of GCP2 (γ-tubulin-complex protein 2) and GCP3 (γ-tubulin-complex protein 3), along with some other proteins [3]. These complexes are usually embedded into MTOCs (microtubule-organizing centres), where they nucleate microtubules. Previous work by ourselves and by others has demonstrated that γ-tubulin is also associated with cellular membranes [4,5] and that protein tyrosine kinases form complexes with γ-tubulin [6,7]. Yet it remains to be clarified what the regulatory mechanisms of microtubule nucleation from membranes are.

Non-receptor Fyn (protein tyrosine kinase p59^{Fyn}), a member of the Src kinase family, has been implicated in events regulating neuronal differentiation and function of neuronal cells. It is highly expressed in developing neurons, and Fyn knockout mice exhibit defects in neurite outgrowth in response to cell-adhesion molecules. However, the knockout of other kinases of the Src family, Src and Yes, does 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 signalling molecules [9].

It has been shown that Src and Lck from the Src kinase family are also involved in the regulation of PI3K (phosphoinositide 3-kinase)-dependent signalling pathways [10] required for neurite outgrowth [11]. Class IA PI3Ks (hereafter referred to as PI3Ks) are heterodimers composed of a p110 catalytic subunit and a regulatory subunit, of which the most extensively studied subunit is p85α (regulatory p85α subunit of PI3K). It consists of an inter-SH (Src homology) 2 domain flanked by two SH2 domains. The SH3 domain and RhoGAP (Rho GTPase-activating protein) domain reside in the N-terminal region of the molecule. Upon stimulation, p85α binds to phosphorylated receptors or adaptor proteins, and the bond relieves the basal inhibition of the catalytic subunit by p85α [12]. Although complexes of p85α with γ-tubulin have been described previously [13,14], the location of the

Abbreviations used: Ab, antibody; Bcr, B-cell receptor; CT, C-terminal; Cy3, indocarbocyanine; DRM, detergent-resistant membrane; EGFP, enhanced green fluorescent protein; Fyn, protein tyrosine kinase p59^{Fyn}; GCP2, γ-tubulin-complex protein 2; GST, glutathione transferase; HD, high density; HEK-293 cell, human embryonic kidney cell; HRP, horseradish peroxidase; LD, low density; mAb, mouse monoclonal antibody; MTOC, microtubule-organizing centre; NP-40, Nonidet P40; P_{20}, pellet from 18 200 rev./min centrifugation; P_{100}, pellet from 40 700 rev./min centrifugation; p85α, regulatory p85α subunit of PI3K; PI3K, phosphoinositide 3-kinase; PN_{100}, P_{100} pellet treated with NP-40; pY418, phospho-Tyr^{418}; pY530, phospho-Tyr^{530}; RA, *all-trans*-retinoic acid; RhoGAP, Rho GTPase-activating protein; S_{20}, supernatant from 18 200 rev./min centrifugation; S_{55}, supernatant from 30 100 rev./min centrifugation; S_{100}, supernatant from 40 700 rev./min centrifugation; SN_{100}, S_{100} treated with NP-40; SH, Src homology, cSH2, C-terminal SH2.

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γ-tubulin-binding site on p85α, as well as the type of this interaction, are unknown. The biological relevance of the γ-tubulin–p85α association is also unclear.

Murine P19 embryonal carcinoma cells offer a well-characterized experimental model for *in vitro* neuronal differentiation induced by treatment with RA (*all-trans*-retinoic acid) [15]. RA stimulation induces changes in the expression of signalling molecules [16] that make these cells suitable for biochemical studies of the early stages of neuronal differentiation. We have shown previously [16] 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 by 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.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail tablets were from Roche. The Src family tyrosine kinase inhibitors PP2 and PP3 (used as a 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, an inhibitor of the tyrosine kinases Bcr (B-cell receptor)-Abl and c-kit, was donated by Novartis. Stock solutions (10 mM) of wortmannin, SU6656, PP2, PP3 and imatinib mesylate were prepared in DMSO. Recombinant PI3K (p110δ complexed to p85α) and Fyn were purchased from Upstate Laboratories. Tubulin was prepared from porcine brain as described previously [17].

DNA constructs

Total RNA was isolated from Balb/c adult mouse brain using TRIzol® reagent (GibcoBRL). Reverse transcription was performed with random hexamers and the SuperScript III Reverse Transcriptase kit (Invitrogen). The fragment encoding mouse GCP2 (GenBank® nucleotide sequence database accession number NM_133755) was amplified by PCR using forward (5′-AGTCGGATCCAGCGAATTTCGGATTCAC-3′) (BamHI restriction site is underlined) and reverse (5′-AAGCGTCGACTCAGCCAATCAGGAAATCTC-3′) (SalI restriction site is underlined) primers, with total cDNA used as the template. The isolated fragment was ligated into pGEX-6P-1 (Amersham Biosciences) for preparation of GST (glutathione transferase)-tagged fusion protein.

Full-length human p85α cDNA was a gift from Dr L. Rameh (Boston Biomedical Research Institute, Watertown, MA, U.S.A.) [18]. Restriction sites were introduced by PCR, and p85α fragments were subcloned into pGEX-6P-1. The fragment encoding the cSH2 (C-terminal SH2) domain of p85α with a mutation (R649L) in the FLVR motif was amplified from the p85-CT R649L construct (where CT is C-terminal) provided by Dr T. O. Chan (Center for Translational Medicine, Thomas Jefferson University, Philadelphia, PA, U.S.A.) [10] and subcloned into pGEX-6P-1 to create cSH2-R649L. For *in vivo* experiments, p85α and its fragments were subcloned into the pCI-NEO (Promega) expression vector. The p85α-CTwt (where wt is wild-type) construct encoding the C-terminal fragment of p85α was provided by Dr. T. O. Chan. Full-length mouse Fyn cDNA was a gift from Dr Petr Dráber [19] (Laboratory of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic), and the FYN–EGFP (where EGFP is enhanced green fluorescent protein) construct was prepared by subcloning Fyn into the pEGFP-N3 vector (Clontech).

Full-length cDNA of human γ-tubulin was obtained from Dr B. Oakley (Department of Molecular Genetics, Ohio State University, Columbus, OH, U.S.A.) [20]. EcoRI and XhoI restriction sites were introduced by PCR, and full-length γ-tubulin was subcloned into the pGEX-6P-1 vector for the production of fusion protein. Constructs were verified by restriction mapping and DNA sequencing. GST-tagged fusion proteins were expressed in *Escherichia coli* strain BL-21 after induction with IPTG (isopropyl β-D-thiogalactoside) and purification on glutathione–Sepharose 4B Fast Flow beads (Amersham Biosciences) according to the manufacturer's instructions.

Antibodies

The rabbit polyclonal Ab (antibody) and mAb (mouse monoclonal Ab) against Fyn were from Santa Cruz Biotechnology and BD Transduction Laboratories respectively. Anti-p85α rabbit polyclonal Ab and HRP (horseradish peroxidase)-conjugated anti-phosphotyrosine mAb 4G10 were from Upstate Laboratories. Anti-p85α mAb was from U.S. Biological. Anti-γ-tubulin mAb (GTU-88), anti-γ-tubulin rabbit polyclonal Abs (T5192 and T3195), anti-β-tubulin mAb (TUB 2.1) and FITC-conjugated anti-β-tubulin mAb (TUB 2.1-FITC) were from Sigma. Anti-GFP polyclonal Ab was from Clontech. Anti γ-tubulin (TU-31; IgG2b), anti-α-tubulin (TU-01) and anti-neurofilament protein (NF-09, IgG2a) mAbs have been described previously [16]. DY547-conjugated anti-γ-tubulin mAb (TU-30), anti-Fyn mAb (Fyn-01) and anti-GFP rabbit polyclonal Ab (for immunoprecipitation of GFP) were from Exbio Praha. The mAb NF-09 and an anti-(non-muscle myosin) rabbit polyclonal Ab (Biomedical Technologies) were used as negative controls for immunoprecipitation experiments. Abs against the catalytic active pY418 (phospho-Tyr^{418}) and inactive pY530 (phospho-Tyr^{530}) forms of Src, recognizing the homologous phosphorylation sites in Fyn, were from BioSource International. Anti-GST rabbit polyclonal Ab was provided by Dr Petr Dráber and anti-centrin mAb (20H5) by Dr J. L. Salisbury (Mayo Clinic, Rochester, MN, U.S.A.) [21]. Secondary HRP-conjugated Abs were from Promega. FITC-conjugated anti-mouse and Cy3 (indocarbocyanine)-conjugated anti-rabbit polyclonal Abs were from Jackson Immunoresearch Laboratories.

Anti-GCP2 rabbit polyclonal Ab was prepared according to a method described previously [22], and was affinity-purified on GST–GCP2 coupled to CNBr-activated Sepharose. The Ab was eluted using 3.5 M $MgCl_2$ in 5 % (v/v) dioxane and dialysed 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 incubating cells with 1 μM RA for 9 days as described previously [16]. In some cases, cells were incubated at 37 °C for 60 min with 20 μM SU6656, 20 μM PP2, 20 μM PP3 or 20 μM imatinib mesylate (an inhibitor of the tyrosine kinases Bcr-Abl and c-kit) before extraction. Alternatively, cells were incubated at 37 °C for 60 min with 200 nM wortmannin, an inhibitor of PI3K, or with 10 mM methyl-β-cyclodextrin (to deplete cholesterol). For immunofluorescence visualization of differentiated cells, a modified cell-culture procedure (in the presence of cytosine β-D-arabinofuranoside) was

used [16]. HEK-293-FT cells (where HEK-293 cell is human embryonic kidney cell) (Promega) were transfected with 2–20 μg of DNA per 6-cm-diameter tissue-culture dish using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

Preparation of whole-cell extracts

All whole-cell extracts were prepared at 4 °C. For differential centrifugation, cells were rinsed with Hepes buffer [50 mM Hepes/NaOH (pH 7.6), 75 mM NaCl, 1 mM $MgCl_2$ and 1 mM EGTA] and scraped in Hepes buffer supplemented with protease and phosphatase (1 mM Na_3VO_4 and 1 mM NaF) inhibitors. Cells were disrupted by sonication (2 × 10 s pulses, amplitude 20) using a 500W Ultrasonic Homogenizer (Cole Parmer), and cell nuclei were removed by centrifugation at 1000 ***g*** for 10 min, whereas post-nuclear supernatants were centrifuged at 18 200 rev./min for 15 min (Beckman MLA-130 rotor). From this, S_{20} (supernatant from 18 200 rev./min centrifugation) fractions were centrifuged at 30 100 rev./min for 30 min (Beckman MLA-130 rotor). The S_{55} (supernatant from 30 100 rev./min centrifugation) fractions were subsequently centrifuged at 40 700 rev./min for 1 h (Beckman MLA-130 rotor) to obtain high-speed S_{100} (supernatant from 40 700 rev./min centrifugation) and P_{100} (pellet from 40 700 rev./min centrifugation) fractions. Alternatively, S_{20} fractions were supplemented with Hepes buffer or with NP-40 (Nonidet P40) to a final concentration of 0.5 %, incubated for 30 min and centrifuged at 40 700 rev./min for 1 h (Beckman MLA-130 rotor). S_{100} or SN_{100} (S_{100} treated with NP-40) fractions were mixed with 5 × SDS sample buffer, whereas the corresponding P_{100} and PN_{100} (P_{100} treated with NP-40) fractions were resuspended in 1 × SDS sample buffer in a volume which was the same as that of the supernatant fractions. In some experiments, the P_{100} fraction was resuspended with Hepes buffer supplemented with one of the following detergents: Brij 98, NP-40 or Triton X-100 (at a final concentration of 1 %). After 30 min incubation, the detergent-insoluble material was centrifuged at 30 100 rev./min for 30 min (Beckman MLA-130 rotor).

Whole-cell extracts for the GST pull-down assays (1 % NP-40 in Hepes buffer) were prepared as described previously [16]. For precipitation from the cytosolic fraction, the S_{100} fraction was adjusted with 1 % NP-40 (final concentration). For precipitation from the microsomal fraction (P_{100}) (hereafter referred to as membranes), the pelleted material was resuspended in Hepes buffer supplemented with 1 % NP-40. An equal volume of buffer to the corresponding supernatant was used for resuspension of the pellet. The suspension was homogenized by sonication (5 s pulse, amplitude 10) and, after 15 min incubation, the suspension was centrifuged at 18 200 rev./min for 15 min (Beckman MLA-130 rotor).

Density-gradient centrifugation

For density-gradient centrifugation, whole-cell extracts were obtained by incubation in ice-cold 1 % Triton X-100 in Hepes buffer supplemented with protease and phosphatase inhibitors for 10 min at 4 °C, followed by centrifugation at 18 200 rev./min for 15 min at 4 °C (Beckman MLA-130 rotor). Where indicated, 0.2 % saponin was added to the extraction buffer. Step-density gradients [10–40 % (w/v) sucrose in Hepes buffer] were prepared in Ultra-Clear tubes (14 × 95 mm, Beckman). Extracts were adjusted to 40 % (w/v) sucrose by adding equal volumes of 80 % (w/v) sucrose in Hepes buffer, and 3 ml of the whole-cell extract was loaded per tube, followed by 7 ml of 30 % (w/v) sucrose and 2 ml of 10 % (w/v) sucrose. Gradients were centrifuged at 35 000 rev./min for 21 h at 4 °C (Beckman SW40 rotor), and fractionated from the top of the tube into 11 fractions. The floating DRMs (detergent-resistant membranes) were concentrated in fraction 2. To concentrate DRMs, this fraction was diluted with 6 vols of ice-cold water and centrifuged at 35 000 rev./min for 1 h at 4 °C (Beckman SW40 rotor). For SDS/PAGE, pelleted DRMs were mixed with SDS sample buffer at 1:30 of the volume of the original fraction. Alternatively, pelleted DRMs were resuspended in BRB80 buffer [80 mM Pipes (pH 6.8), 1 mM $MgCl_2$ and 1 mM EGTA] and used for the microtubule spin-down assay.

Immunoprecipitation, kinase assay and immunoblotting

Immunoprecipitation was performed as described previously [16]. Protein A–sepharose beads were saturated with: (i) anti-Fyn Ab, (ii) anti-p85α Ab, (iii) anti-GFP Ab, (iv) anti-(non-muscle myosin) Ab (negative control), (v) anti-γ-tubulin mAb (TU-31), (vi) NF-09 mAb (negative control) or (vii) immobilized Protein A alone. For comparison of immunoprecipitation from unstimulated and stimulated cells, whole-cell extracts were adjusted to the same protein concentration. Alternatively, beads with immunoprecipitated material were used for the *in vitro* kinase assay as described previously [7]. The ^{32}P-labelled-immunocomplexes were separated by SDS/PAGE (7.5 % gels), transferred on to nitrocellulose membranes and the ^{32}P-labelled proteins were detected using the bioimaging analyser BAS-5000 (Fuji Photo Film).

Immunoblotting was performed as described previously [23]. Anti-tubulin mouse mAbs TU-01 (obtained from tissue-culture medium in which hybridoma cells secreting the TU-01 Ab were cultured), TUB 2.1 and GTU-88 were used at 1:50, 1:2000 and 1:5000 dilutions respectively. Anti-Fyn, anti-p85α, anti-centrin and anti-phosphotyrosine mAbs were used at 1:300, 1:500, 1:1000 and 1:30 000 dilutions respectively. Anti-GFP and anti-GCP2 Abs were used at 1:500 dilution, whereas the anti-GST Ab was used at 1:30 000 dilution. Anti-pY418 and anti-pY530 Abs were both used at 1:5000 dilution. Secondary HRP-conjugated Abs were used at 1:10 000 dilution and detection was performed using chemiluminescence reagents (Supersignal West Pico; Pierce).

GST pull-down assay

GST pull-down assays with whole-cell extracts were performed as described previously [16]. Alternatively, sedimented beads with immobilized GST–γ-tubulin were incubated with PI3K at a concentration of 0.4 μg/ml in TBS [Tris buffered saline; 10 mM Tris/HCl (pH 7.4) and 150 mM NaCl]. After washing, bound proteins were eluted by boiling for 5 min in SDS sample buffer. In some experiments, 0.1 μg of active Fyn was added to the beads with immobilized GST-fusion proteins and subjected to a kinase assay (see below).

Immune complex PI3K assay

PI3K activity was determined essentially as described previously [24]. After immunoprecipitation, the beads were washed in kinase buffer and resuspended in kinase buffer with or without 100 nM wortmannin. After 10 min incubation at 25 °C, the assay was initiated by the addition of kinase buffer containing sonicated phosphatidylinositol and [γ-^{32}P]ATP, and the reaction mixture was incubated at 25 °C for 30 min.

Microtubule spin-down assay and immunofluorescence

Membranes were washed in BRB80 buffer and resuspended in BRB80 buffer by sonication (5 s pulse, amplitude 10). The spin-down assay was performed as described previously [25].

Membranes were incubated with 10 μM tubulin in the presence of 1 mM GTP for 20–30 min at 37 °C. After fixation with 1 % glutaraldehyde, membranes were centrifuged through a 10 % (v/v) glycerol cushion on to glass coverslips, fixed in cold methanol and used for immunostaining. Alternatively, concentrated DRMs were used for the assay. In salt-washed experiments, the membranes were first incubated in 2 M KCl for 1 h on ice, then centrifuged at 30 100 rev./min for 30 min at 4 °C (Beckman MLA-130 rotor), extensively washed in BRB80 buffer at 4 °C and finally resuspended by sonication in BRB80 buffer. In recovery experiments, salt-washed membranes were incubated for 1 h at room temperature (24 °C) with cytosol (S_{100}) preabsorbed with a negative control Ab or with cytosol immunodepleted of γ-tubulin. After washing in BRB80 buffer, the samples were used in the assay. Immunodepletion of γ-tubulin was performed by incubation of 600 μl of cytosol (diluted 1:5 in Hepes buffer) with Protein A–Sepharose beads saturated with a mixture of polyclonal Abs against γ-tubulin. After 45 min incubation at room temperature, the beads were centrifuged at 2000 ***g*** for 30 s and the supernatant was subjected to three additional subsequent absorption steps with new Ab-saturated beads. The same procedure was performed with a negative control Ab.

Membrane preparations after the spin-down assay were stained for tubulin using TUB 2.1-FITC (1:50 dilution). TUB 2.1-FITC Ab and DY547-conjugated anti-γ-tubulin mAb (TU-30) (1:500 dilution) were used for double labelling. Alternatively, samples were stained with TUB 2.1-FITC, anti-Fyn (1:500 dilution) or anti-p85α (1:2000 dilution) Abs, followed by a Cy3-conjugated anti-rabbit Ab (1:500 dilution).

Immunofluorescence microscopy on fixed cells was performed as described previously [23]. Cells were fixed in 3 % (w/v) formaldehyde and extracted with 0.5 % Triton X-100. The samples were double labelled with an anti-γ-tubulin rabbit Ab (1:500 dilution) and an anti-Fyn mAb (Fyn-01, 1:100 dilution), followed by an Cy3-conjugated anti-rabbit Ab and an FITC-conjugated anti-mAb (1:200 dilution). Staining was examined using an Olympus A70 Provis microscope. Treatment with the conjugated secondary Abs alone did not result in any detectable staining (results not shown).

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 homogenates revealed that γ-tubulin associated with both the pellet [P_{20} (pellet from 18 200 rev./min centrifugation), P_{55} (pellet from 30 100 rev./min centrifugation) and P_{100}] and the supernatant (S_{20}, S_{55} and S_{100}) fractions. On the other hand, centrin, a marker of centrosomes, was localized exclusively to the low-speed P_{20} fraction (Figure 1A).

To determine the relative distribution of tubulins and kinases, the low-speed S_{20} fraction was supplemented with or without 0.5 % NP-40 before centrifugation at 40 700 rev./min for 60 min (Beckman MLA-130 rotor). In the absence of detergent, γ-tubulin was equally distributed between the cytosolic (S_{100}) and membrane (P_{100} pellet) fractions. In contrast, β-tubulin and p85α predominated in the cytosolic fraction, whereas Fyn prevailed mainly in the membrane fraction (Figure 1B). Distribution of α-tubulin resembled that of β-tubulin (results not shown). γ-Tubulin associated with the detergent-resistant fraction (PN_{100}) even in the presence of 0.5 % NP-40. Under such conditions, β-tubulin prevailed mainly in the detergent-soluble fraction (SN_{100}). The distribution of Fyn and p85α in SN_{100} and PN_{100} fractions

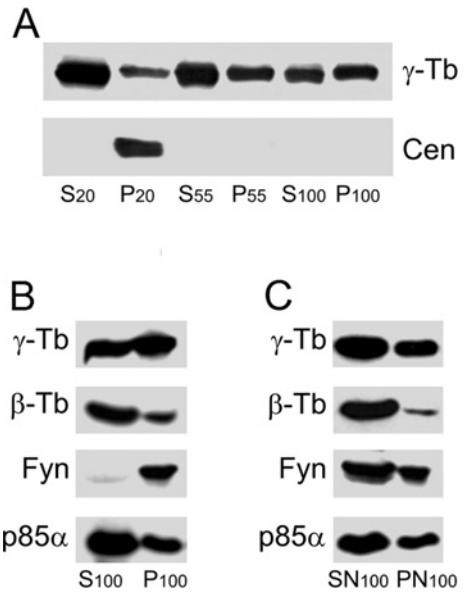

Figure 1 Distribution of γ-tubulin and kinases in RA-differentiated P19 cells

(**A**) Relative distribution of γ-tubulin (γ-Tb) and centrin (Cen) in fractions after differential centrifugation of whole-cell extracts. Cell fractions were prepared as described in the Materials and methods section. (**B**) Immunoblotting of S_{100} and P_{100} fractions with Abs against γ-tubulin (γ-Tb), β-tubulin (β-Tb), Fyn and p85α. (**C**) Immunoblotting of SN_{100} and PN_{100} fractions after centrifugation in the presence of 0.5 % NP-40. To compare the relative distribution of proteins, pelleted material was resuspended in a volume equal to that of the corresponding supernatant.

resembled that of γ-tubulin (Figure 1C). When membrane-associated γ-tubulin (P_{100} pellet) was extracted with lysis buffer containing 1 % Brij 98, 1 % NP-40 or 1 % Triton X-100, approx. 50 %, 45 % and 40 % γ-tubulin respectively was still associated with the detergent-resistant material. These results indicate that the distribution patterns of γ-tubulin and $\alpha\beta$-tubulin dimers differ, and that a substantial proportion of γ-tubulin is present in the cellular fraction containing Fyn. A proportion of γ-tubulin associated with the 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. Co-localization of Fyn and γ-tubulin was observed in some regions of thicker processes (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/416/bj4160421add.htm).

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

An *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 μM $\alpha\beta$-tubulin dimer and 1 mM GTP. The spin-down assay revealed that microtubules grew from membranes (Figure 2A, panels a, c and e) that contained γ-tubulin (Figure 2A, panel b), Fyn (Figure 2A, panel d) as well as p85α (Figure 2A, panel 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 of the membrane fragments stained for β-tubulin after incubation with tubulin dimers, as similarly reported previously for isolated Golgi membranes [4], but only some displayed nucleated microtubules.

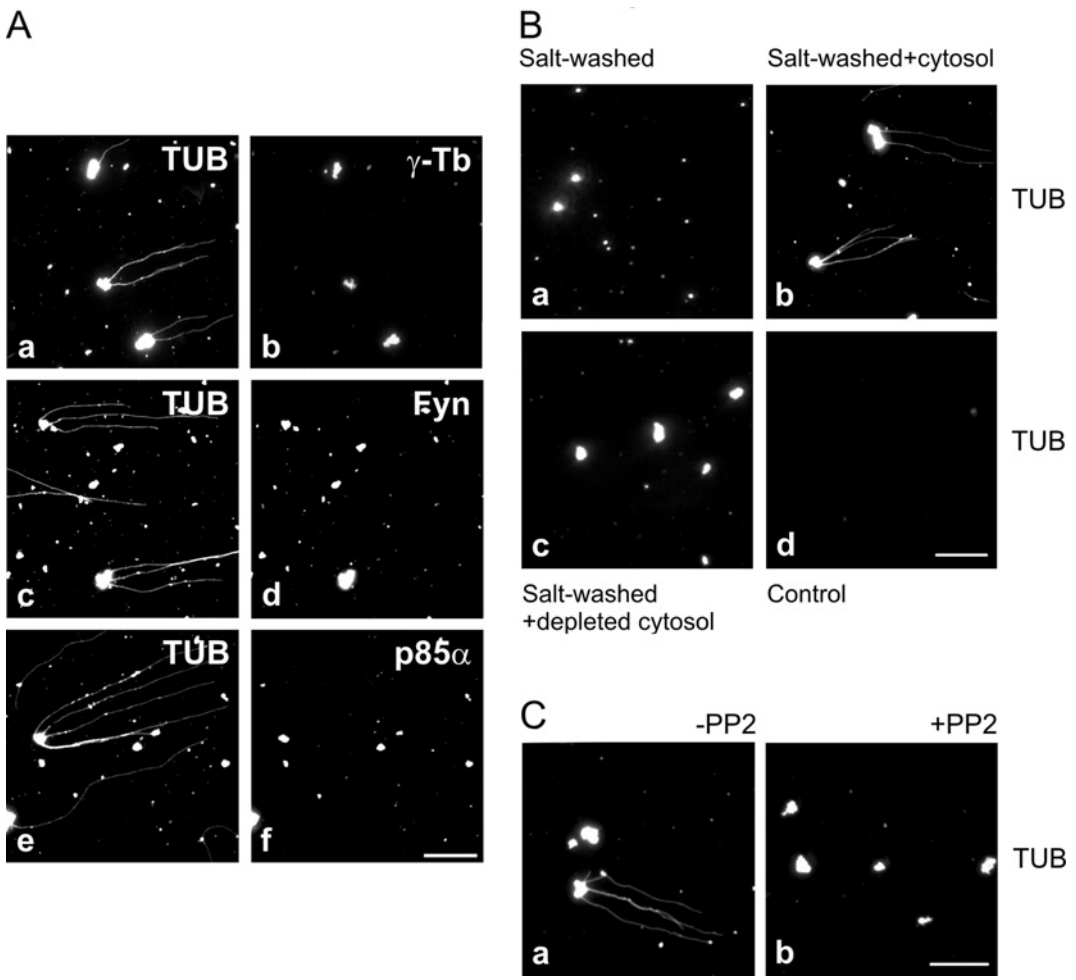

Figure 2 Assembly of microtubules from membranes

Isolated membranes were incubated for 20 min at 37 °C with 10 μM tubulin and 1 mM GTP. After fixation, samples were centrifuged on to coverslips and used for immunostaining. (**A**) Double-label staining of membranes with assembled microtubules. Staining of β-tubulin (TUB) (panels a, c and e), γ-tubulin (γ-Tb) (panel b), Fyn (panel d) and p85α (panel f). (**B**) Staining of β-tubulin (TUB) (panels a–d) in salt-washed membranes (panel a), salt-washed membranes incubated with cytosol (panel b) and salt-washed membranes incubated with cytosol immunodepleted of γ-tubulin (panel c). A negative control (without membranes) is also shown (panel d). (**C**) Membranes from control cells (− PP2) (panel a) or cells pretreated with 20 μM PP2 (+ PP2) (panel b) were incubated with tubulin and after a spin-down assay were stained for microtubules using an Ab against β-tubulin (TUB). Scale bar, 10 μm in all panels.

To determine whether γ-tubulin is responsible for microtubule nucleation from membranes, isolated membranes were treated with 2 M KCl. Under these conditions, nucleation was not detected (Figure 2B, panel a). In recovery experiments, salt-treated membranes were incubated with cytosol preabsorbed with a negative control Ab prior to *in vitro* microtubule-assembly assay. In this case, nucleation of microtubules was restored (Figure 2B, panel b). When salt-treated membranes were incubated with cytosol immunodepleted of γ-tubulin, nucleation of microtubules was absent (Figure 2B, panel c). No spontaneous microtubule assembling took place when $\alpha\beta$-tubulin dimer was incubated in the absence of membranes (Figure 2B, panel d). Immunoblotting experiments confirmed a substantially higher amount of γ-tubulin in untreated membranes when compared with salt-washed membranes. γ-Tubulin staining was observed after incubation of salt-washed membranes with cytosol preabsorbed with a 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 S2 at http://www.BiochemJ.org/bj/416/bj4160421add.htm).

When cells were pretreated with the Src family kinases inhibitors SU6556 or PP2 (20 μM), microtubule nucleation was inhibited (Figure 2C, panel b). Nucleation was not affected when DMSO alone was used (Figure 2C, panel a). It is known that PP2 also inhibits the tyrosine kinases c-kit and Bcr-Abl, in addition to Src family kinases [26]. Nucleation experiments were therefore performed with membranes isolated from cells pretreated with 20 μM imatinib mesylate (which inhibits c-kit and Bcr-Abl). In this case, no inhibition was observed (results not shown). Pretreatment of cells with 200 nM wortmannin also inhibited microtubule nucleation on isolated membranes (see Supplementary Figure S3A at http://www.BiochemJ.org/bj/416/bj4160421add.htm). Pretreatment with wortmannin or PP2 did not affect the association of γ-tubulin with membranes (see Supplementary Figure S3B). These *in vitro* experiments suggest that γ-tubulin associated with the microsomal fraction has the capability to nucleate microtubules, and that this process depends on the activity of Src family kinases and PI3K.

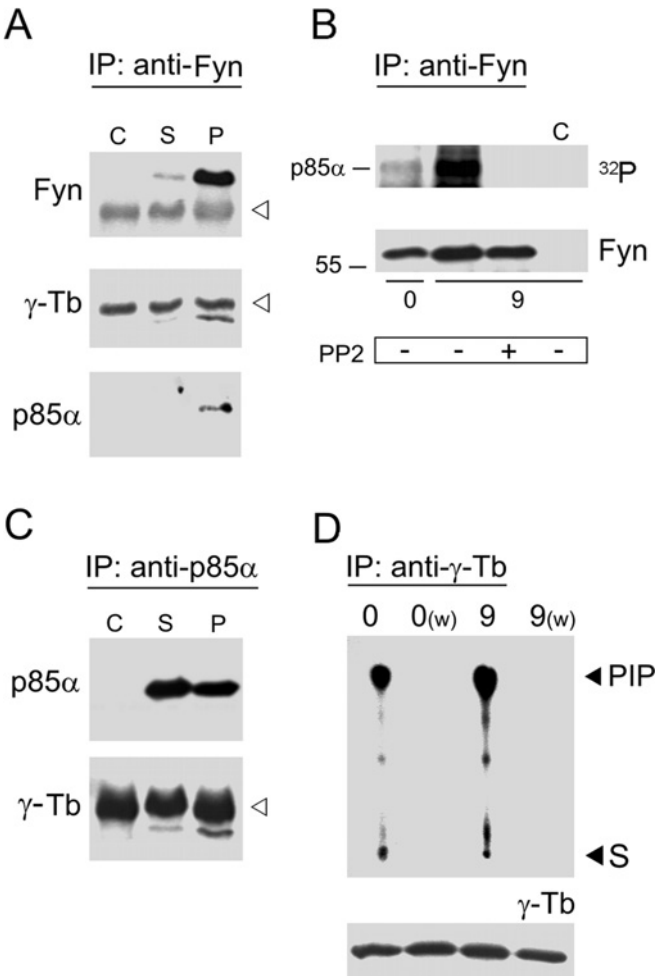

Figure 3 Co-immunoprecipitation of membrane-bound γ-tubulin with Fyn and p85α

(**A**) Soluble (S) and membrane (P) fractions from differentiated cells were immunoprecipitated (IP) with an Ab against Fyn and blots were probed with Abs against Fyn, γ-tubulin (γ-Tb) and p85α. Immobilized Ab not incubated with whole-cell extract was used as a control (C). IgG heavy chain is indicated (arrowheads). (**B**) Kinase activity in Fyn membrane immunocomplexes immunoprecipitated from resting (0) and differentiated (9) cells pretreated with (+) or without (−) 20 μM PP2, and after immunoprecipitation with a negative control Ab (C). Immunoprecipitated Fyn was detected by immunoblotting. Positions of p85α and molecular-mass marker (in kDa) are indicated on the left-hand side. (**C**) Soluble (S) and membrane (P) fractions from differentiated cells were immunoprecipitated with an Ab against p85α and the blots were probed with Abs against p85α and γ-tubulin (γ-Tb). As a control, the immobilized Ab not incubated with whole-cell extract is shown (C). IgG heavy chain is indicated (arrowheads). (**D**) Activity of PI3K in γ-tubulin membrane immunocomplexes immunoprecipitated with an anti-γ-tubulin Ab (anti-γ-Tb) from resting (0) and differentiated (9) cells treated with (w) or without wortmannin. Immunoprecipitated γ-tubulin was detected by immunoblotting with an anti-γ-tubulin Ab (γ-Tb). The migration of phosphatidylinositol phosphate (PIP) and the starting point of thin-layer chromatography (S) are noted.

Membrane-associated γ-tubulin interacts with Fyn and p85α

A series of precipitation experiments was performed to find out whether γ-tubulin and Fyn form complexes in membrane fractions of differentiated P19 cells. An anti-Fyn Ab allowed co-immunoprecipitation of Fyn with γ-tubulin as well as p85α (Figure 3A). After immunoprecipitation with a negative control Ab, no specific staining was detected with Abs against Fyn, γ-tubulin and p85α. As Fyn formed complexes with p85α, we intended to find out whether p85α could serve as a substrate for Fyn in P19 cells. An *in vitro* kinase assay revealed protein phosphorylation in Fyn immunocomplexes isolated from membranes from both resting and differentiated cells. A protein corresponding to p85α, as confirmed by immunoblotting with a 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). However, treatment with PP3 had no effect (results not shown). No kinase activity was detected after precipitation with a negative control Ab (Figure 3B).

Complexes of γ-tubulin with p85α have been described previously in cells overexpressing the insulin receptor [13,14]. Therefore we next examined the possibility that membrane-bound γ-tubulin associates with p85α in differentiated P19 cells. Immunoprecipitation using an Ab against p85α resulted in the co-immunoprecipitation of γ-tubulin, and the amount was higher in immunocomplexes from the membrane fraction (Figure 3C). To corroborate the results of the immunoblotting experiments, PI3K activity was also evaluated by the immune complex kinase assay. PI3K activity associated with membrane-bound γ-tubulin was detected in both resting and differentiated cells. However, a 1.82 ± 0.24-fold (mean $\pm$ S.D., $n = 3$) 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 (phosphatidylinositol phosphate) (Figure 3D). In summary, these results demonstrate that membrane-bound γ-tubulin in differentiated P19 cells forms complexes with both Fyn and active PI3K.

γ-Tubulin associates with DRMs

To determine whether γ-tubulin is also associated with DRMs, cells were solubilized in 1% Triton X-100 and lysates were fractionated by equilibrium-density-gradient centrifugation. In order to compare the results, the same amounts of protein were loaded for centrifugation and the corresponding blots were exposed on the same autoradiography film. Fyn served as a marker for DRMs. Immunoblotting experiments revealed substantial differences in the distribution of γ-tubulin as a result of the time course of RA-induced differentitation. In undifferentiated cells (day 0), both Fyn and γ-tubulin were only found in HD (high density) fractions, whereas in differentiated cells (day 9), they were detected in both HD and LD (low density) fractions (Figure 4A). LD fractions correspond to floating DRMs, whereas HD fractions correspond to fractions not associated with DRMs. Since DRMs are rich in cholesterol, differentiated cells were extracted with buffer containing 1% Triton X-100 and 0.2% saponin, which is known to sequester cholesterol. Both Fyn and γ-tubulin were absent in LD fractions in saponin-treated samples (Figure 4A). A 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 whole-cell extracts and DRMs. The amounts of protein in the whole-cell extract (Figure 4B, lanes 1) corresponded to those in concentrated DRMs (Figure 4B, lanes 2). γ-Tubulin was present in DRMs at a relatively high concentration in comparison with β-tubulin. Importantly, GCP2, a component of γ-tubulin complexes, was detected in DRMs. As expected, Fyn was highly enriched in DRMs. The microtubule-nucleation capacity of concentrated DRMs was demonstrated by a spin-down assay. Microtubules grew from DRMs (Figure 4C, panel a) that also contained γ-tubulin (Figure 4C, panel b). Immunoprecipitation experiments with an anti-Fyn Ab, followed by immunobloting with an Ab against γ-tubulin confirmed an association of γ-tubulin with Fyn in DRMs (results not shown). Collectively, these results indicate

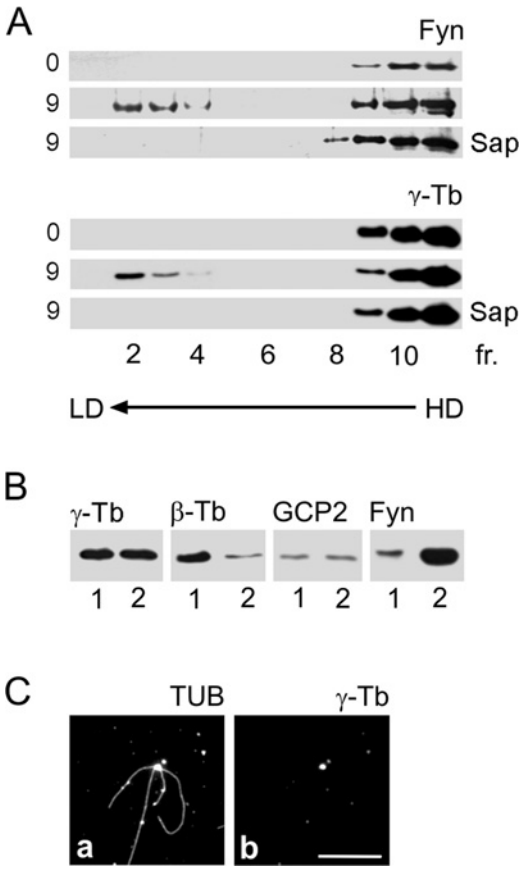

Figure 4 Distribution of Fyn and γ-tubulin during density-gradient centrifugation of lysates from resting and RA-differentiated P19 cells

Cells were extracted in lysis buffer with 1 % (v/v) Triton X-100 in the absence or presence of the 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 against Fyn and γ-tubulin (γ-Tb). Numbers 0 and 9 indicate days of incubation of cells with RA. Numbers at the bottom indicate individual fractions (fr.). (**B**) Relative distribution of proteins in whole-cell extracts (lanes 1) and concentrated DRMs (lanes 2). Immunoblotting was performed using Abs against γ-tubulin (γ-Tb), β-tubulin (β-Tb), GCP2 and Fyn. The same amount of protein (0.3 μg) was loaded into each lane. (**C**) Assembly of microtubules from DRMs. Concentrated DRMs were incubated with tubulin and after spin-down were double-label stained for β-tubulin (TUB) (panel a) and γ-tubulin (γ-Tb) (panel b). Scale bar, 10 μm.

that a subpopulation of γ-tubulin is associated with DRMs and there forms complexes with Fyn.

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

To answer the question of whether the association of Fyn with membranes is important for its interaction with γ-tubulin, an 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 alone (used as a control) was found exclusively in the soluble fraction (Figure 5A). An anti-γ-tubulin Ab precipitated γ-tubulin from both fractions and coprecipitated membrane-targeted Fyn–EGFP, but not cytosolic Fyn–EGFP or EGFP only (Figure 5A). An anti-phosphotyrosine Ab stained neither soluble nor membrane-bound γ-tubulin in immunoprecipitates (results not shown). This

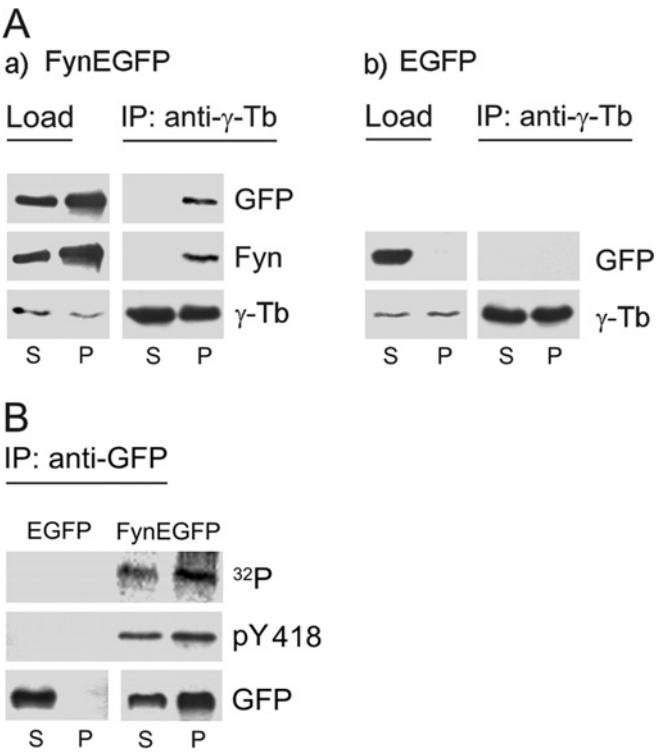

Figure 5 Association of γ-tubulin with Fyn in cells expressing EGFP-tagged Fyn

(**A**) Soluble (S) and membrane (P) fractions from cells expressing Fyn–EGFP (a) and EGFP (b) were immunoprecipitated (IP) with an Ab against γ-tubulin (anti-γ-Tb) and blots of whole-cell extracts (Load) and immunoprecipitated proteins were probed with Abs against GFP, Fyn and γ-tubulin (γ-Tb). (**B**) Soluble (S) and membrane (P) fractions from cells expressing EGFP and Fyn–EGFP (FynEGFP) proteins were immunoprecipitated with an Ab against GFP. Kinase activity in GFP immunocomplexes (^{32}P) and immunoblots with Abs against the catalytic active form of Fyn (pY418) and GFP are shown. To compare the relative distribution of proteins, the pellet fraction was resuspended in a volume equal to that of the corresponding supernatant.

suggests that γ-tubulin is not phosphorylated by Fyn. To rule out the possibility that cytosolic Fyn–EGFP is not folded properly and therefore is not accessible for the interaction with γ-tubulin, we used the *in vitro* kinase assay as a measure of proper folding. Membrane and cytosolic Fyn–EGFP were precipitated by an anti-GFP Ab and the immunoprecipitated material was used for the *in vitro* kinase assay. Both membrane and cytosolic Fyn–EGFP precipitates were phosphorylated and contained an active form of Fyn, as demonstrated by autoradiography and by immunoblotting with an Ab against the autophosphorylation site (pY418 Ab) (Figure 5B). Although an Ab against the negative regulatory site (pY530 Ab) also reacted with the membrane and cytosolic forms of Fyn–EGFP, non-active Fyn–EGFP did not accumulate in the cytosol (results not shown). These combined results suggest that both membrane and cytosolic Fyn–EGFP contains a mixture of active and non-active forms of the 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 cSH2 domain of p85α, outside the tyrosine-phosphorylated protein-recognition site

Because γ-tubulin associates with p85α in differentiated P19 cells, we attempted to identify the region in p85α which is responsible for the interaction with γ-tubulin. The pull-down assays with GST-tagged p85α domains (Figure 6A) and cell extracts from differentiated cells revealed that γ-tubulin associates with all

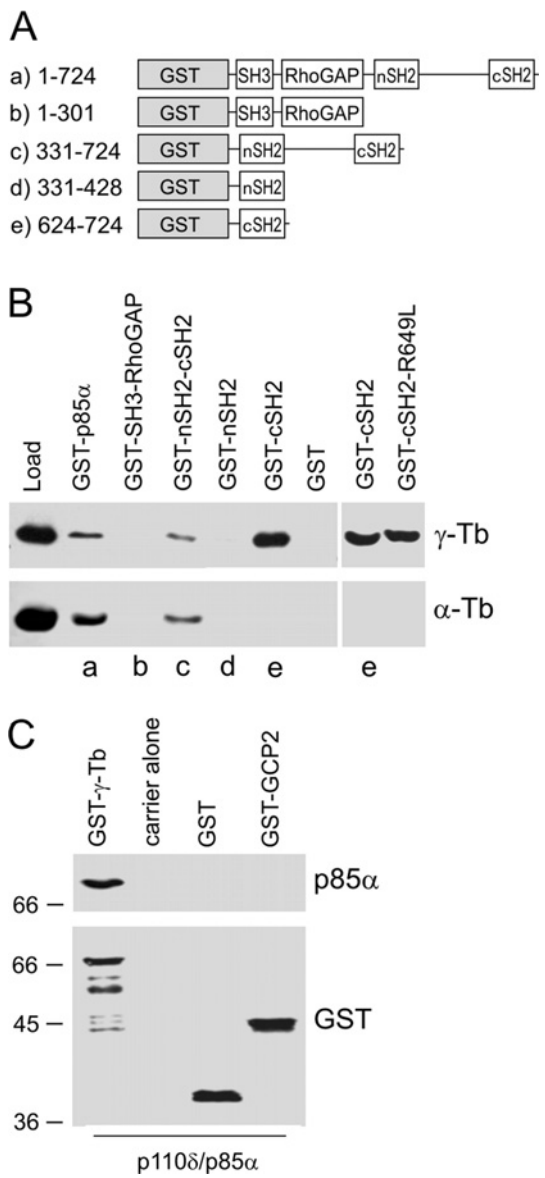

Figure 6 Interaction of γ-tubulin with p85α domains in GST pull-down assay

(**A**) Schematic diagram of GST–p85α (a) and its truncated forms (b–e) used in the pull-down assays. Values at the left-hand side indicate the residues of full-length p85α present in the truncated construct. (**B**) Whole-cell lysates from differentiated cells (Load) were incubated with GST-fusion protein constructs [a, b, c, d or e as in (**A**)] or GST alone immobilized on glutathione–Sepharose beads. Immunoblots of bound proteins were probed with Abs against γ-tubulin (γ-Tb) and α-tubulin (α-Tb). (**C**) Binding of PI3K to GST–γ-tubulin. PI3K (p110δ complexed to p85α) was incubated with immobilized GST–γ-tubulin (GST–γ-Tb), GST alone or GST–GCP2 fragment. Alternatively, PI3K was incubated with beads without GST-fusion proteins (carrier alone). Immunoblots of bound proteins were probed with Abs against p85α (p85α) or GST. Molecular-mass markers (in kDa) are indicated on the left-hand side. nSH2, N-terminal SH2 domain.

the fusion proteins containing the cSH2 domain. No interaction was observed with fusion proteins containing SH3–RhoGAP fragment, the nSH2 (N-terminal SH2) 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 made evident by staining with an anti-GST Ab (results not shown). These results indicate that the association of γ-tubulin with the cSH2 domain is independent of the presence of the $\alpha\beta$-tubulin dimer. All SH2 domains contain a conserved FLVR motif, which is essential for phosphotyrosine binding. Mutation of the critical arginine residue in the FLVR motif abolishes the capability of SH2 domain to bind phosphotyrosine proteins [27]. When the pull-down assay was performed with fusion protein containing the mutated arginine residue in the cSH2 domain (GST–cSH2-R649L), the binding of γ-tubulin to this fusion protein was preserved (Figure 6B). To decide whether γ-tubulin interacts with p85α directly, the GST pull-down assay was performed with GST–γ-tubulin and recombinant PI3K (p110δ complexed to p85α). Though PI3K did bind to GST–γ-tubulin, it failed to bind to GST fusion protein containing a fragment of GCP2 (negative control) or GST alone (Figure 6C). The amounts of immobilized GST fusion proteins present in each pull-down were similar (Figure 6C).

To prove that the cSH2 domain is responsible for γ-tubulin binding *in vivo*, p85α and its truncated forms (Figure 7A) were transfected into HEK-293 cells. All constructs were expressed in the cells (Figure 7B) and did not alter the expression of γ-tubulin (Figure 7B). Whole-cell extracts were precipitated with an Ab against γ-tubulin and blots were probed with an Ab against p85α. As expected, the amount of p85α in complex with γ-tubulin increased after overexpression of p85α. However, the truncated forms of p85α without the cSH2 domain (p85-ΔCT and p85-inter-ΔCT, see Figure 7A) were absent in γ-tubulin immunocomplexes. In contrast, p85α with a deleted RhoGAP domain, but with an intact cSH2 domain, was immunoprecipitated (Figure 7B). When the C-terminal fragment of p85α containing the cSH2 domain was expressed in cells, a dose-dependent decrease in p85α detected in γ-tubulin immunocomplexes was evident (Figure 7C), whereas the expression level of p85α and γ-tubulin was unchanged (Figure 7C). Collectively, these results 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 p85α.

DISCUSSION

It is known that the bulk of cytoplasmic γ-tubulin is not associated with MTOCs [28]. In the present study, we show that γ-tubulin is equally distributed to both soluble and membrane fractions in RA-differentiated P19 cells. A fraction of membrane-associated γ-tubulin was resistant to non-ionic detergents. However, $\alpha\beta$-tubulin dimers prevailed in the soluble pool. Interestingly, γ-tubulin in differentiated cells was found not only in rough membrane fractions, but also in DRMs. 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 DRMs compared with 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 DRMs [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 the membrane fraction. First, Fyn was associated with γ-tubulin in immunoprecipitates from membranes. Secondly, complexes of both proteins were present in DRMs that are known to accumulate Fyn. Thirdly, γ-tubulin and Fyn were located on membranes from which microtubules were nucleated. Fourthly, the nucleation activity

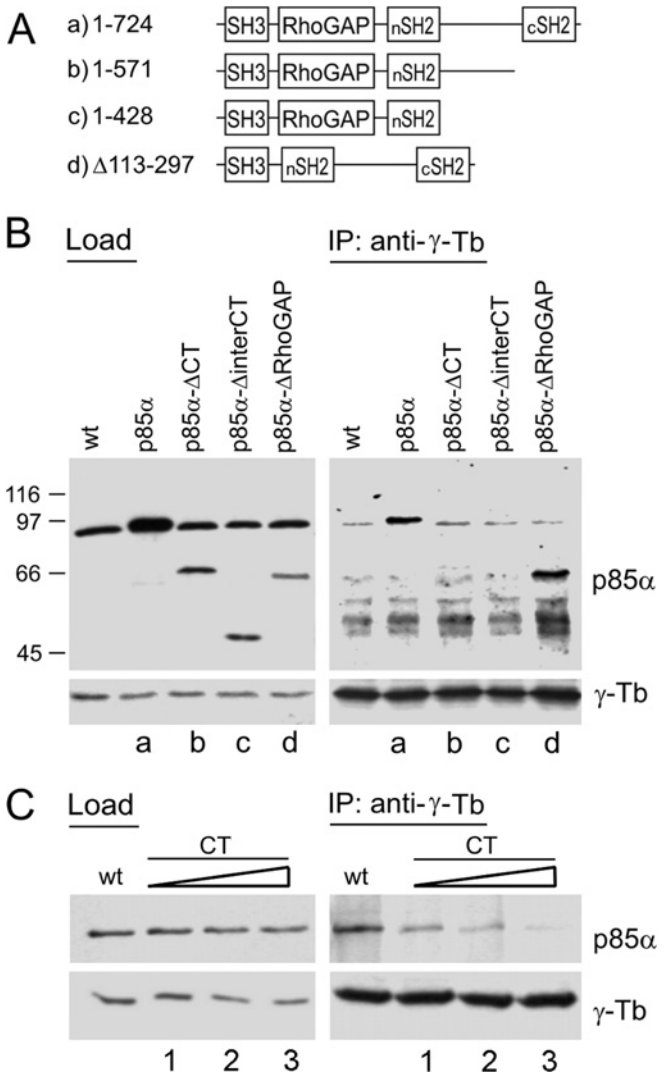

Figure 7 Interaction of γ-tubulin with p85α truncated forms expressed in cells

(**A**) Schematic diagram of p85α (a) and its truncated forms (b–d) used for expression in HEK-293 cells. (**B**) Whole-cell extracts [wild-type cells (wt), cells expressing exogenous p85α (a), p85α-ΔCT (b), p85α-ΔinterCT (c) or p85α-ΔRhoGAP (d)] (Load) were immunoprecipitated (IP) with an Ab against γ-tubulin (anti-γ-Tb) and blots of both whole-cell extracts and immunoprecipitates were stained with Abs against p85α and γ-tubulin (γ-Tb). Molecular-mass markers (in kDa) are indicated on the left-hand side. (**C**) Whole-cell extracts (Load) of HEK-293 cells transfected with increasing amounts of DNA encoding p85α CT (CT) were immunoprecipitated with an Ab against γ-tubulin (γ-Tb). Blots of whole-cell extract and immunoprecipitates were stained with Abs against p85α and γ-tubulin (γ-Tb). Wild-type cells (wt) and cells transfected with DNA [5 μg (lane 1), 10 μg (lane 2) and 20 μg (lane 3)].

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 the modulation of microtubule nucleation from membranes. Finally, tests with EGFP-tagged Fyn showed that association of Fyn with membranes was required for the 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 an increase in activity of PI3K associated with γ-tubulin. Immunoprecipitation experiments confirmed the association of γ-tubulin with p85α. Although *in vitro* interactions between γ-tubulin and p85α have been reported [13,14], no conclusions were drawn on the exact location of the γ-tubulin-binding site on p85α and the type of interaction occurring between these two proteins. Our results from GST pull-down assays with p85α domains demonstrate that γ-tubulin binds to the cSH2 domain and that phosphotyrosine-mediated binding is not required for this interaction. Moreover, the GST pull-down assay with the γ-tubulin fusion protein and recombinant PI3K confirms a direct interaction. Immunoprecipitation experiments from transfected cells expressing truncated forms of p85α or overexpressing the cSH2 domain demonstrate that the cSH2 domain is essential for the interaction of p85α with γ-tubulin *in vivo*. The capability of the cSH2 domain of p85α to mediate a phosphorylation-independent interaction has been reported for the serine/threonine kinase A-Raf [30], and the same may hold true for γ-tubulin.

It has been shown that the cSH2 domain of p85α regulates the activity of PI3K. This domain carries the Tyr^{688} residue that can be phosphorylated by cytoplasmic kinases [10]. Phosphorylation of this site then functionally blocks the inhibitory effect of p85α on the catalytic subunit. Neutralization of the inhibitory domain renders p85α 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 knockout mice [31]. In the present study, we show that, in P19 cells, Fyn can phosphorylate p85α *in vivo* and that this phosphorylation is enhanced in differentiated cells. The efficacy of Fyn in phosphorylating p85α was further confirmed by an *in vitro* kinase assay with GST–cSH2 and purified active Fyn (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/416/bj4160421add.htm). This indicates that Fyn in P19 cells phosphorylates the cSH2 domain of p85α and thus regulates the activity of the catalytic subunit.

The results obtained from the microtubule spin-down assay show that membrane-associated γ-tubulin complexes can nucleate microtubules from both rough membrane fractions and DRMs. In this context, 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 DRMs are most abundant in the plasma membrane, they first assemble in the Golgi apparatus [33]. It is therefore uncertain whether microtubule formation from DRMs reflects nucleation from the plasma membrane or from other membranous components.

Our results 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 results indicating that upregulation of Fyn leads to Fyn accumulation in membranes and phosphorylation of p85α. It could lead to activation of the p110 catalytic subunit and initiation of the PI3K signalling pathway. Recruitment of p85α with bound γ-tubulin to 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 signalling pathway, could participate in the modulation of microtubule nucleation.

In conclusion, the present study shows that in differentiating P19 cells, γ-tubulin is associated with membranes, including DRMs. Membrane-associated γ-tubulin is involved in the 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 the cSH2 domain of p85α. The findings suggest that signalling-cascade

proteins could modulate non-centrosomal microtubule nucleation by membrane-associated γ-tubulin.

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online data

SUPPLEMENTARY ONLINE DATA

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

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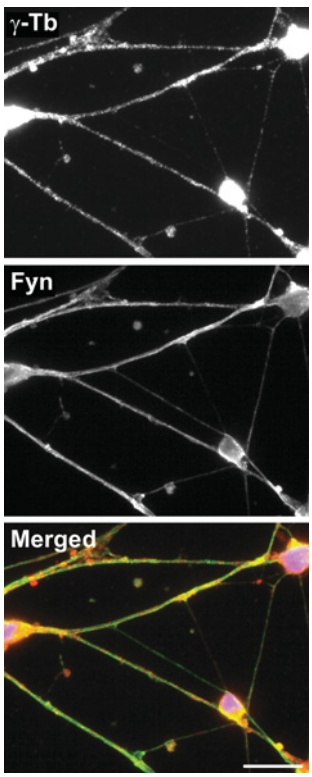

Figure S1 Immunofluorescence localization of γ-tubulin and Fyn in differentiated P19 cells

Double-label staining with Abs against γ-tubulin (γ-Tb) (red) and Fyn kinase (green). DAPI (4′,6-diamidino-2-phenylindole) was used to label cell nuclei (blue in merged image). Scale bar, 20 μm.

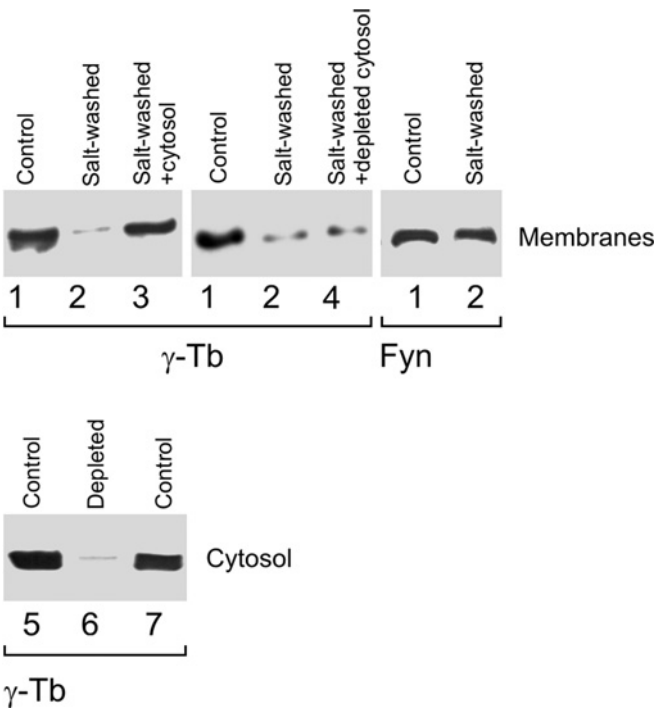

Figure S2 Distribution of γ-tubulin and Fyn in membrane fractions used in microtubule spin-down assay

Immunoblot analysis of isolated membrane fractions (lanes 1–4) and cytosolic fractions (lanes 5–7). Lane 1, control membranes (Control). Lane 2, salt-washed membranes. Lane 3, salt-washed membranes incubated with cytosol pretreated with negative control Ab (salt-washed + cytosol). Lane 4, salt-washed membranes incubated with cytosol immunodepleted of γ-tubulin (salt-washed + depleted cytosol). Lane 5, control cytosol (Control). Lane 6, cytosol after γ-tubulin depletion (Depleted). Lane 7, cytosol after incubation with negative control Ab (Control). Blots were stained with Abs against γ-tubulin (γ-Tb) or Fyn.

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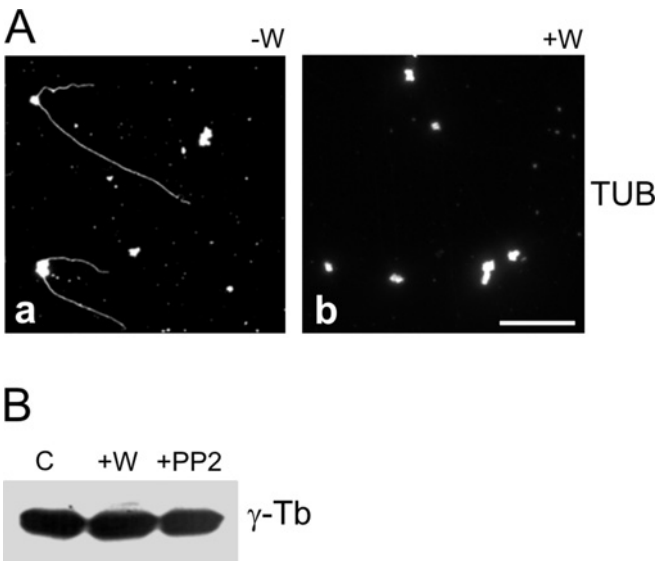

Figure S3 Effect of wortmannin on assembly of microtubules from membranes

(**A**) Membranes from control cells (panel a) (−W) or cells pretreated with 200 nM wortmannin (b) (+W) were incubated with tubulin and after a microtubule spin-down assay were stained for microtubules with an Ab against β-tubulin (TUB). Scale bar, 10 μm. (**B**) Immunoblotting of isolated membranes from control cells (C), cells pretreated with 200 nM wortmannin (+W) or 20 μM PP2 (+PP2) with an Ab against γ-tubulin (γ-Tb).

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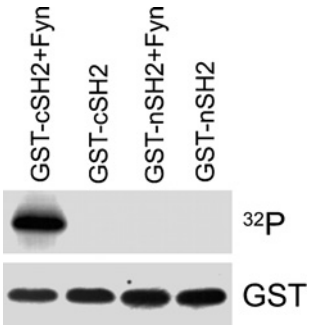

Figure S4 Tyrosine phosphorylation of the cSH2 domain of p85α with Fyn

GST-fusion proteins immobilized on glutathione–Sepharose beads were used for an *in vitro* kinase assay with purified Fyn. The bound proteins were eluted into SDS sample buffer and separated by SDS/PAGE. The presence of GST-fusion proteins was confirmed by immunoblotting with an Ab against GST. nSH2, N-terminal SH2 domain.