Regulation of Microtubule Formation in Activated Mast Cells by Complexes of γ-Tubulin with Fyn and Syk Kinases[1]

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Aggregation of the high-affinity IgE receptors (FcεRIs) on the surface of granulated mast cells initiates a chain of signaling events culminating in the release of allergy mediators. Although microtubules are involved in mast cell degranulation, the molecular mechanism that controls microtubule rearrangement after FcεRI triggering is poorly understood. In this study, we show that the activation of bone marrow-derived mast cells (BMMCs) induced by FcεRI aggregation or treatment with pervanadate leads to a rapid polymerization of microtubules. This polymerization was not dependent on the presence of Lyn kinase as determined by experiments with BMMCs isolated from Lyn-negative mice. One of the key regulators of microtubule polymerization is γ-tubulin. Immunoprecipitation experiments revealed that γ-tubulin from activated cells formed complexes with Fyn and Syk protein tyrosine kinases and several tyrosine phosphorylated proteins from both wild-type and Lyn$^{-/-}$ BMMCs. Pretreatment of the cells with Src-family or Syk-family selective tyrosine kinase inhibitors, PP2 or piceatannol, respectively, inhibited the formation of microtubules and reduced the amount of tyrosine phosphorylated proteins in γ-tubulin complexes, suggesting that Src and Syk family kinases are involved in the initial stages of microtubule formation. This notion was corroborated by pull-down experiments in which γ-tubulin complex bounds to the recombinant Src homology 2 and Src homology 3 domains of Fyn kinase. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins, and thus modulate the microtubule nucleation in activated mast cells. *The Journal of Immunology,* 2006, 176: 7243–7253.

Granulated mast cells play a pivotal role in allergy and inflammation. Their granules contain inflammatory mediators such as histamine, proteases, lipid mediators, and cytokines. Mast cells express on their surfaces receptors with a high affinity for IgE (FcεRI). An aggregation of the FcεRI by multivalent Ag-IgE complexes triggers a series of biochemical events leading to fusion of cytoplasmic granules with the plasma membrane and release of the inflammatory mediators (1). The first defined steps in FcεRI signaling are activation of protein tyrosine kinases of the Src family (Lyn and Fyn) and Syk/Zap family and phosphorylation of their substrates (2). Two signaling pathways have been discovered in FcεRI-activated mast cells. One involves sequential activity of Lyn and Syk kinases and tyrosine phosphorylation of the FcεRI and the linker for activation of T cells. Phosphorylated linker for activation of T cells then serves as an anchor for binding of phospholipase Cγ, which is crucial in generating increased levels of intracellular calcium (3). The second pathway uses Fyn kinase, which is required for FcεRI-induced phosphorylation of Gab2 and for mast cell degranulation, but not for a rapid enhancement of intracelular calcium concentration (4). Mast cells can also be activated by an exposure to pervanadate, a compound that inhibits protein tyrosine phosphatases; such activation also leads to the secretion of inflammatory mediators (5).

Microtubules play an important role in mast cell degranulation, as the movement of secretory granules depends on intact microtubules (6) and agents inhibiting tubulin polymerization suppress the degranulation (7–9). Recently, Nishida et al. (10) documented that FcεRI stimulation triggered the formation of microtubules and that drugs affecting microtubule dynamics effectively suppressed the FcεRI-mediated translocation of granules to the plasma membrane and the degranulation. Furthermore, the translocation of granules to the plasma membrane occurred in a calcium-independent manner, whereas the release of mediators and granule-plasma membrane fusion were completely dependent on calcium. Thus, the degranulation process can be dissected into two events: the calcium-independent microtubule-dependent translocation of granules to the plasma membrane and calcium-dependent membrane fusion and exocytosis. The same authors also showed that the Fyn/Gab2/RhoA (but not Lyn/SLP-76) signaling pathway played a critical role in the calcium-independent microtubule-dependent pathway (10). Although these data confirmed that a dynamic microtubule network is required for mast cell degranulation, the precise roles of tyrosin kinases and the molecular mechanisms controlling microtubule rearrangements in this process are still unknown.

One of the key components required for microtubule formation is γ-tubulin (11), a highly conserved member of the tubulin superfamily that is located on the minus end of microtubules in microtubule organizing center (12). Interestingly, the majority of γ-tubulin is, however, associated with other proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complex (γ-TuRC)[3]

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[3] Abbreviations used in this paper: γ-TuRC, γ-tubulin-ring complex; RBL, rat basophilic leukemia; BMMC, bone marrow-derived mast cell; SH, Src homology; TNP,

(13, 14) is formed by small complexes (γ-tubulin small complex) (15), comprising two molecules of γ-tubulin, one molecule each of γ-tubulin complex proteins 2 and 3 (16, 17), and some other proteins. In addition to the nucleation from microtubule organizing center, γ-TuRCs are also involved in the regulation of the dynamics of microtubule minus ends (18). γ-Tubulin itself has been recognized as a microtubule minus-end binding molecule in nonanchored microtubules (19). Soluble γ-tubulin can associate with αβ-tubulin dimers irrespective of the size of γ-tubulin complexes (20). γ-Tubulin has also been found to bind to membranous components of the cell (21–23). Several reports indicated that kinases might be involved in the regulation of γ-tubulin interactions (24–28).

We have previously shown that γ-tubulin formed complexes with tyrosine-phosphorylated proteins and Lyn kinase in rat basophilic leukemia (RBL) cells activated by FcεRI aggregation or by an exposure to pervanadate (29). To decide whether Lyn kinase is indispensable for the formation of such complexes, we analyzed interactions of γ-tubulin with kinases and their substrates in resting and activated bone marrow-derived mast cells (BMMCs) isolated from wild-type or Lyn-deficient mice. The data indicate that fully functional microtubule nucleation is attained even in the absence of Lyn kinase. We have found that γ-tubulin in Lyn$^{-/-}$ BMMCs formed complexes with Fyn and Syk kinases, which could thus have a key role in γ-tubulin-mediated microtubule nucleation in activated mast cells.

Materials and Methods

Reagents

Immobilized Protein A Plus and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce and protease-inhibitor mixture tablets ("Complete EDTA-free") were from Roche Molecular Biochemicals. Phenyl phosphate, phosphoserine, the Syk kinase-selective inhibitor piceatannol, the Src-family selective tyrosine kinase inhibitor SU6656 and isopropyl β-D-1-thiogalactopyranoside were obtained from Sigma-Aldrich. The Src-family selective tyrosine kinase inhibitor PP2 and the PP3 (negative control for inhibitor PP2) were obtained from Calbiochem. [γ-^{32}P]ATP (110 TBq/mmol; 370 MBq/ml) and glutathione Sepharose 4B were from Amersham Biosciences. Synthetic peptides were prepared at the Institute of Biochemistry and Organic Chemistry, Czech Academy of Sciences or at Sigma-Genosys. The primers were from Genetica. Tubulin was prepared from porcine brain (30), and associated proteins were removed by phosphocellulose chromatography (31).

DNA constructs

Total cellular mRNA was obtained from mouse Neuro2a cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (32). Reverse transcription was performed with random hexamers (Amersham Biosciences) and Moloney murine leukemia virus-reverse transcriptase (Invitrogen Life Technologies). The fragment coding both Src homology (SH)3 and SH2 domains (aa 80–245) of mouse Fyn kinase (EMBL, accession no. M27266) was amplified by PCR using forward 5′-GAACTC CTCCTCTCACACTGGGACC-3′ and reverse 5′-CTTTAGCCAATCCA GAAGTTTGTGGG-3′ primers and total cell cDNA as a template. Isolated fragment was directly ligated into pCR3.1 vector (Invitrogen Life Technologies) and isolated DNA was subsequently used as a template for preparation of constructs encoding the GST-tagged fusion proteins. pFYSH2 vector was constructed by PCR amplification of SH2 domain of Fyn kinase (aa 80–143) using forward 5′-GACGGATCCTGGTACTTTGGAA AACTTG-3′ and reverse 5′-AGCCTCGAGTCATGAAACCACAGTT AAG-3′ primers, restriction by BamHI and XhoI and ligation into pGEX-6P-1 vector (Amersham Biosciences). pFYSH3 vector was constructed by PCR amplification of SH3 domain of Fyn kinase (aa 148–245) using forward 5′-GCAGAATTCGGGACAGGAGTGACAC-3′ and reverse 5′-AT ACCTCGAGATTCAGATGGAGTCAACTGG-3′ primers, restriction by EcoRI and XhoI and subsequent ligation into pGEX-6P-1 vector. All constructs were verified by restriction analyze and bidirectional sequencing

with universal pGEX5′ and pGEX3′ primers (Amersham Biosciences). GST-tagged fusion proteins were expressed in *Escherichia coli* strain BL21 after isopropyl β-D-1-thiogalactopyranoside induction.

Cells

Mouse BMMCs and Lyn$^{-/-}$ BMMCs were provided by M. Hibbs (Ludwig Institute for Cancer Research, Melbourne, Australia) (33). The cells were incubated in suspension cultures in freshly prepared culture medium (RPMI 1640 supplemented with 20 mM HEPES (pH 7.5), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM MEM nonessential amino acids, 1 mM sodium pyruvate) supplemented with 10% FCS and 10% WEHI-3 cell supernatant as a source of IL-3. Cells were grown at 37°C in 5% CO$_2$ in air and passaged every 2 days. No discernible differences in growth properties and morphology were detected between BMMCs and Lyn$^{-/-}$ BMMCs. In some cases, cells intended for preparation of extracts for immunoprecipitation were pretreated for 60 min at 37°C with Src family selective tyrosine kinase inhibitors SU6656, PP2, and/or PP3 (negative control for PP2) at concentrations of 5–20 μM, and piceatannol at concentration of 10–50 μM.

Antibodies

Polyclonal Abs to p53/p56^{lyn} (Lyn-44), 59^{fyn} (FYN3), Fgr, and Zap were from Santa Cruz Biotechnology. mAbs to p59^{fyn} (clone 25, IgG2b,), Lck (IgG2a), Yes (IgG1), and phosphotyrosine (PY-20, IgG2b,) were obtained from BD Transduction Laboratories. Polyclonal Abs to Hck, phosphotyrosine and mAb to phosphotyrosine (4G10, IgG1) labeled with HRP were from Upstate Laboratories. mAb to pp60^{src} (clone 327, IgG1) was from Oncogene Research Products. Abs Lyn-01/Pr (IgG1) to p53/p56^{lyn} (34), Syk-01 (IgG1), and rabbit Ab against Syk (35) were described previously. mAbs to human γ-tubulin peptide 38-53 (GTU-88, IgG1), α-tubulin (DM1A, IgG1), β-tubulin (TUB 2.1, IgG1), phosphoserine (PSR-45, IgG1) phosphothreonine (PTR-8, IgG2b) and polyclonal Ab to actin were from Sigma-Aldrich. Abs TU-31 (lgG2b) and TU-32 (IgG1) to human γ-tubulin peptide 434–449 (36), TU-01 (lgG1) to α-tubulin and TU-06 (IgM) were specified previously (37). Immunofluorescence was conducted with polyclonal Ab TUB to αβ-tubulin dimer (38) and Ab TUB 2.1 labeled with indocarbocyanate (Cy3). α-Tubulin on immunoblots was detected with polyclonal Ab to tyrosinated α-tubulin (39). Ab IGEL b4 1 (IgE), specific for 2,4,6-trinitrophenyl (TNP) (40), was used to sensitize the cells. Abs NF-09 (IgG2a) (41), VI-01 (IgM) (42) and polyclonal Ab to nonmuscle myosin (Biomedical Technologies) served as negative controls. Ab against GST was prepared by immunizing rabbits with GST. Anti-mouse Abs and anti-rabbit Abs conjugated with HRP were purchased from Promega Biotec. Cy3-conjugated anti-mouse and FITC-conjugated anti-rabbit Abs were from Jackson ImmunoResearch Laboratories. FITC-conjugated anti-mouse Ab specific for IgG was from Sigma-Aldrich.

Cell activation and preparation of cell extracts

Cells were harvested, resuspended in culture medium at concentration 10 × 10^6 cells/ml, and sensitized in suspension with TNP-specific mAb IGEL b4 (IgE; ascitic fluid diluted 1/1000) for 60 min at 37°C. The cells were then centrifuged, washed twice in buffered saline solution (BSS) containing 20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 5.6 mM glucose, 1 mM MgCl$_2$, and 0.1% BSA (BSS-BSA), and activated for 1–6 min with the cross-linking reagent TNP-BSA at a final concentration of 1 μg/ml. Alternatively, the cells were activated by pervanadate. Pervanadate solution was freshly made by mixing sodium orthovanadate solution with hydrogen peroxide to get a 10 mM final concentration of both components. The pervanadate solution was incubated at room temperature for 15 min and then diluted 1/100 into cell suspension in BSS-BSA. Cells were incubated for 3 or 15 min at 37°C. Cell activation was stopped by transferring the tubes on ice and pelleting the cells by a brief centrifugation. For immunofluorescence experiments, cells were attached to poly-L-lysine-covered coverslips and thereafter sensitized with IgE Ab and activated with Ag for 3 min. Alternatively, attached cells were incubated with pervanadate solution for 3 or 15 min.

Whole cell extracts for SDS-PAGE were prepared by washing the cells in cold MES buffer (100 mM MES adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM MgCl$_2$), solubilizing them in hot SDS-sample buffer (43) without bromphenol blue and boiling for 5 min.

For evaluation of protein distribution into soluble and detergent-resistant fractions under microtubule depolymerizing conditions, 6.5 × 10^6 cells were rinsed twice in cold MES buffer and then extracted with 0.4 ml of cold MES buffer supplemented with protease inhibitor mixture, phosphatase inhibitors (1 mM Na$_3$VO$_4$, 1 mM NaF) and 1% Nonidet P-40. After 30 min incubation at 4°C, the suspension was spun down at 20,000 × g for 15 min at 4°C, and the same volume of 2× SDS-PAGE sample buffer was added

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2,4,6-trinitrophenyl; BSS, buffered saline solution; MSB, microtubule-stabilizing buffer.

to the supernatant. The insoluble material in the pellet was gently rinsed twice with MES buffer containing inhibitors, resuspended in 0.4 ml of MES buffer with inhibitors and mixed with 0.4 ml of $2\times$ SDS-PAGE sample buffer.

For analysis of microtubule polymer in resting and activated cells, 6.5×10^6 cells were rinsed twice in MES buffer at 37°C and then extracted with 0.4 ml of MES buffer supplemented with protease and phosphatase inhibitors, 2 M glycerol and 0.2% Triton X-100. After a 2-min incubation at 37°C, the suspension was spun down at $8,000 \times g$ for 15 min at 25°C; the nuclear pellet containing cytoskeleton was resuspended in SDS-PAGE sample buffer.

When preparing the extract for immunoprecipitation and for binding to immobilized GST-fusion proteins, cells were rinsed twice in cold MES buffer and extracted at a concentration 15×10^6 cells/ml for 10 min at 4°C with MEM buffer supplemented with protease inhibitor mixture, phosphatase inhibitors and 1% Nonidet P-40. The suspension was then spun down ($20,000 \times g$, 15 min, 4°C), and supernatant collected. Protein quantification in SDS-PAGE-samples was performed by silver dot assay (44) using BSA as a standard.

Immunoprecipitation

Immunoprecipitation was performed as described (45), using TBST (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20) for dilution of extracts and for washings. Cell extracts were incubated with beads of protein A saturated with: 1) rabbit Ab against Fyn kinase, 2) rabbit Ab against phosphotyrosine, 3) rabbit Ab against Syk kinase, 4) negative control rabbit Ab against nonmuscle myosin, 5) mouse Ab TU-31 against γ-tubulin, 6) negative control mouse Ab NF-09, or with 7) immobilized protein A alone. Abs against Fyn, myosin and phosphotyrosine were used at Ig concentration 4 μg/ml. Ab against Syk was used at dilution 1/250. Ab TU-31 and control Ab NF-09 were prepared by mixing 0.1 ml of 10× concentrated hybridoma supernatant with 0.9 ml of the TBST buffer. The washed beads with bound Abs were incubated under rocking for 2 h at 4°C with 1 ml of sample, prepared by diluting the cell extract with TBST at a ratio 1:1. The beads were washed, followed by boiling in SDS-sample buffer to release the bound proteins. Alternatively, beads were washed twice in TBST and further processed in the kinase assay (see below).

Binding of cell extracts to GST fusion proteins

GST fusion proteins were noncovalently coupled to glutathione Sepharose beads (50 μl of sedimented beads) and used after washing in TBST for binding analysis. Sedimented beads were incubated under rocking for 2 h at 4°C with 1 ml of sample, prepared by diluting the cell extract with TBST in the ratio 1:1. Unbound material was removed by four washes in cold TBST, and bound proteins were eluted by boiling in SDS-sample buffer. In competitive inhibition experiments, phenyl phosphate or phosphoserine were added to cell extracts at concentrations varying from 2 to 40 mM before adding the beads, and the mixtures were incubated for 1 h followed by washing and elution as indicated above. When synthetic peptides were used in inhibition experiments, they were used at a concentration ranging from 0.005 up to 5 mM.

In vitro kinase assay

Beads with immunoprecipitated material were washed twice in kinase buffer (25 mM HEPES (pH 7.2), 5 mM MgCl$_2$, 1 mM NaF, 0.1% Nonidet P-40), and resuspended in 30 μl of kinase buffer supplemented with 370 kBq of $[\gamma$-^{32}P]ATP. After incubation for 30 min at 37°C, the reaction was stopped by washing the beads four times in cold kinase buffer and the labeled immunocomplexes were solubilized by boiling for 5 min in 50 μl of SDS-sample buffer. The samples (10 μl) were resolved by SDS-PAGE, transferred to nitrocellulose, and the ^{32}P-labeled proteins were detected using the bioimaging analyzer BAS-5000 (Fuji Photo Film). In some experiments, 5 μg of porcine brain tubulin or 5 μg of BSA were added to the immunocomplexes before kinase assay.

Gel electrophoresis and immunoblotting

SDS-PAGE on 7.5% gels, electrophoretic transfer of separated proteins onto nitrocellulose and details of the immunostaining procedure have been described elsewhere (46). The anti-tubulin Abs TU-01 and TU-32, in the form of spent culture supernatants, were diluted 1/10, whereas GTU-88 was diluted 1/5,000. mAbs against kinases Src, Fyn, Lyn, Syk, and phosphotyrosine (4G10-HRP) were diluted 1/300, 1/250, 1/1,000, 1/1,000 and 1/30,000, respectively. Rabbit Abs against phosphotyrosine, actin, and GST were diluted 1/2,000, 1/2,000 and 1/10,000, respectively. Bound Abs were detected after incubation of the blots with secondary Abs diluted 1/10,000, and after washing with chemiluminiscence reagents in accor-

dance with the manufacturer's directions. Exposed autoradiography films were quantified by densitometry.
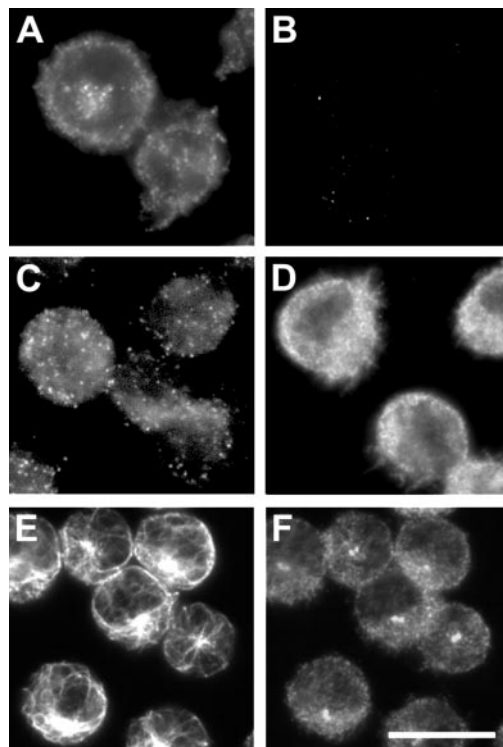
Immunofluorescence

Immunofluorescence microscopy was performed on fixed cells as described (45). Shortly, cells were attached to poly-L-lysine covered coverslips, rinsed briefly with microtubule-stabilizing buffer (MSB; MES buffer supplemented with 4% polyethylene glycol 6000), fixed for 20 min in 3% formaldehyde in MSB and extracted for 4 min with 0.5% Triton X-100 in MSB. Ab TUB against $\alpha\beta$-tubulin dimer was diluted 1/10 and Cy3-conjugated TUB2.1 Ab against β-tubulin was diluted 1/500. Anti-Lyn Ab Lyn-01/Pr and anti-Syk Ab Syk-01 were used as ascitic fluids diluted 1/200, anti-Fyn mAb and anti-γ-tubulin Ab GTU-88 were diluted 1/50 and 1/500, respectively. Anti-phosphotyrosine Ab PY-20 was used at concentration of 2.5 μg/ml. Cy3-conjugated and FITC-conjugated anti-mouse Abs were diluted, respectively, 1/1,000 and 1/100. FITC-conjugated rabbit Ab was diluted 1/200. For double-label staining of microtubules and γ-tubulin, the coverslips were incubated simultaneously with GTU-88 and polyclonal TUB Ab. After washing, the coverslips were incubated simultaneously with the secondary fluorochrome-conjugated Abs. For double-label staining of microtubules and tyrosine phosphorylated proteins, the coverslips were incubated with PY-20 Ab, followed by incubation with FITC-conjugated anti-mouse Ab specific for IgG. The remaining binding sites on FITC-conjugated Ab were blocked by incubation with normal mouse serum (diluted 1/10) before incubation with Cy3-conjugated TUB2.1 Ab. The preparations were mounted in MOWIOL 4–88 (Calbiochem) and examined with Olympus A70 Provis microscope. Conjugates alone did not give any detectable staining.

Results

Distribution of kinases and tubulins in Lyn$^{-/-}$ BMMCs

To compare the expression profiles of protein tyrosine kinases of the Src and Syk/Zap families and tubulins in wild-type and Lyn$^{-/-}$ BMMCs, blots of whole cell extracts were probed with Abs against Src family kinases Lyn, Fyn, Src, Yes, Fgr, Hck, and Lck, Abs against kinases Syk and Zap, and with Abs against tubulins. In both wild-type and Lyn$^{-/-}$ BMMCs, kinases Fyn, Src, and Syk were easily detectable, while Yes, Lck, and Zap kinases were not detected. As expected, Lyn kinase was found only in wild-type cells. A substantially lower signal was detected for Src kinase in comparison to Fyn kinase. From the remaining tested Src family kinases, Fgr and Hck were stained very faintly only after a longer exposure of the film. When Abs against Syk kinase, α-tubulin (55 kDa) and γ-tubulin (48 kDa) were used, similar signal was observed in wild-type and Lyn$^{-/-}$ BMMCs, and the same was true for actin and vinculin. The absence of Lyn kinase in Lyn$^{-/-}$ BMMCs was also confirmed by immunofluorescence microscopy. Dot-like staining concentrated often in pericentrosomal region was detected in wild-type cells with anti-Lyn Ab (Fig. 1A), while no specific staining was detected in Lyn$^{-/-}$ BMMCs (Fig. 1B). In both wild-type (data not shown) and Lyn$^{-/-}$ BMMCs, Fyn kinase (Fig. 1C) and Syk kinase (Fig. 1D) also exhibited dot-like distribution. Double-labeling in Lyn$^{-/-}$ BMMCs revealed that Ab against $\alpha\beta$-tubulin dimer stained a typical network of microtubules originating from centrosomes (Fig. 1E), while the Ab against γ-tubulin stained centrosomes and diffusely the cytoplasm (Fig. 1F). It should be noted that a comparable staining pattern of γ-tubulin was observed with two different mAbs, GTU-88 and TU-31 directed against peptides from N-terminal domain and C-terminal domain of γ-tubulin, respectively, and with polyclonal Ab.

Because γ-tubulin, Fyn kinase, and Syk kinase exhibited a dot-like staining pattern suggesting an association with membrane components, we investigated their distribution in detergent-soluble and insoluble fractions. Extraction of wild-type BMMCs with 1% Nonidet P-40 at 4°C for 30 min showed that there were differences in solubility of Fyn kinase, Syk kinase, and tubulins (Fig. 2). Although Fyn kinase was present in soluble and insoluble fractions in
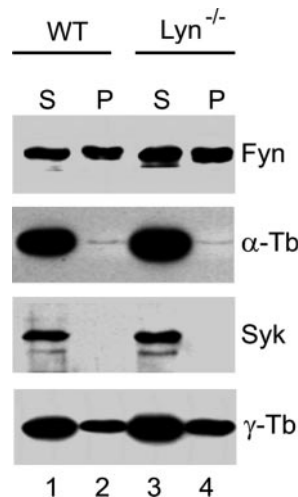
FIGURE 1. Immunofluorescence localization of kinases and tubulins in wild-type (*A*) and Lyn$^{-/-}$ BMMCs (*B–F*). The cells were stained with Abs specific for Lyn (*A* and *B*), Fyn (*C*), and Syk (*D*). Double-label staining with a polyclonal Ab against αβ-tubulin dimers (*E*) and mAb against γ-tubulin (*F*). Pair (*E* and *F*) represents the same cells. The cells were fixed in formaldehyde and extracted in Triton X-100 before labeling. Scale bar, 10 μm. Comparable magnifications are in *A–F*.

FIGURE 2. Immunoblot analysis of soluble and insoluble fractions from wild-type and Lyn$^{-/-}$ BMMCs. To compare the relative distribution of various proteins in wild-type (WT; *lanes 1–2*), and Lyn$^{-/-}$ BMMCs (*lanes 3–4*), the cells were solubilized in lysis buffer with 1% Nonidet P-40, and after centrifugation the supernatant (S) and pellet (P) were separated. Pelleted material was resuspended in a volume equal to the volume of the supernatant. Immunostaining of two identical blots with Abs against Fyn kinase (Fyn) and α-tubulin (α-Tb), and Syk kinase (Syk) and γ-tubulin (γ-Tb). A typical result from three experiments performed.

similar amounts, Syk kinase was found almost exclusively in soluble fraction. α-Tubulin was also highly abounding in soluble fraction. The relative distribution of γ-tubulin resembled that of Fyn kinase, and 38 ± 2% (mean ± SD; $n = 6$) of γ-tubulin was present in insoluble form. A similar distribution pattern of the proteins was found in Lyn$^{-/-}$ BMMCs. These data demonstrate that cells extracted under conditions favoring depolymerization of microtubules contain a significant fraction of both Fyn kinase and γ-tubulin in the detergent-resistant fraction. Thus, based on Nonidet P-40 detergent solubility, γ-tubulin differs from α-tubulin.

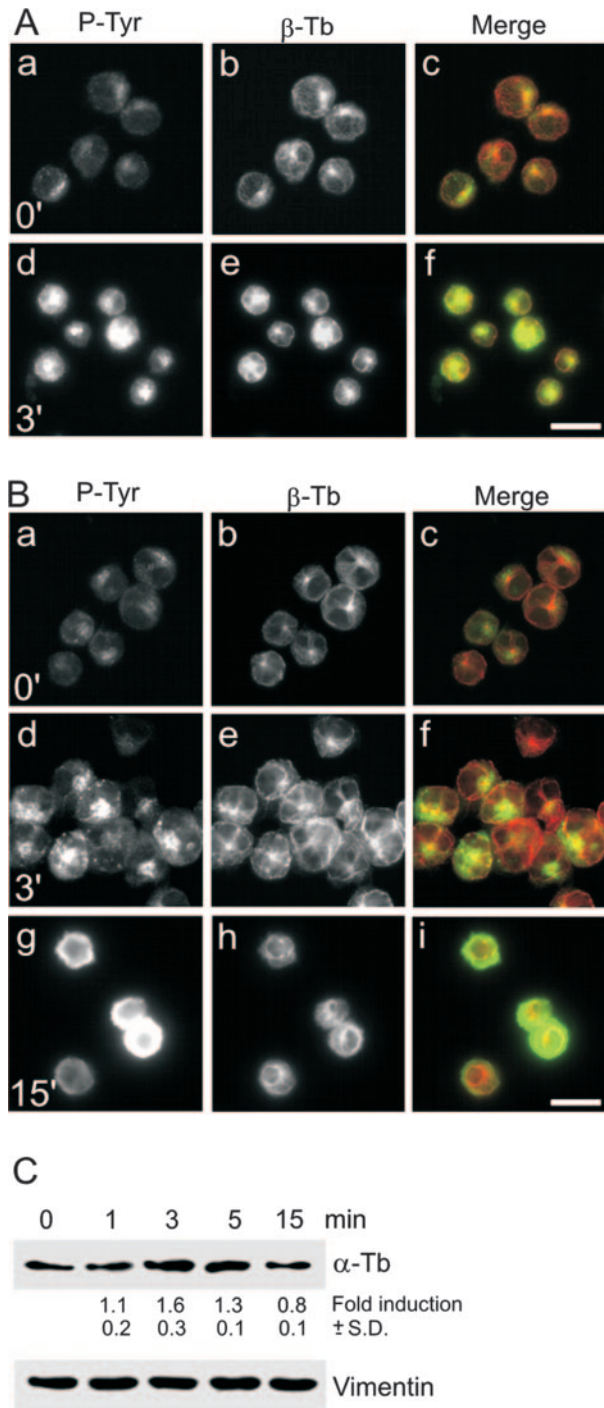*Tyrosine phosphorylation of γ-tubulin-associated proteins in activated cells*

To evaluate the effect of cell activation on microtubule distribution, wild-type and Lyn$^{-/-}$ BMMCs were stimulated by FcεRI aggregation or pervanadate exposure before immunofluorescence double labeling with anti-β-tubulin and anti-phosphotyrosine Abs. Fig. 3*A* shows double-label immunofluorescence experiment on resting and FcεRI-activated Lyn$^{-/-}$ BMMCs with Abs against phosphotyrosine and β-tubulin. In resting cells, tyrosine-phosphorylated proteins were stained very faintly and diffusely in cytoplasm (Fig. 3*Aa*), whereas in cells activated for 3 min with Ag they were stained strongly and were concentrated in the perinuclear region (Fig. 3*Ad*). After activation more intense staining of microtubules was detected (Fig. 3*Ae*) when compared with resting cells (Fig. 3*Ab*). Similar changes in the distribution pattern of tyrosine-phosphorylated proteins and microtubules were observed in wild-type BMMCs (data not shown). Fig. 3*B* shows a similar experiment using Lyn$^{-/-}$ BMMCs activated by pervanadate. Again, in cells activated for 3 min, phosphorylated proteins were concen-

trated in perinuclear region (Fig. 3*Bd*). After 15-min activation, a substantial increase in the staining of proteins phosphorylated on tyrosine was detected at the cell periphery (Fig. 3*Bg*). As to microtubules, 3-min activation resulted in enhanced accumulation of microtubules in cell periphery (Fig. 3*Be*). Longer stimulation (15 min) reduced the staining of microtubules when compared with activation for shorter time (Fig. 3*Bh*). When the cells were pretreated before activation with Src family specific inhibitor PP2, phosphorylated proteins were stained faintly, comparably to nonactivated cells, and staining of microtubules was not increased (data not shown). Quantitative immunoblotting revealed that the amount of polymerized tubulin was increasing with a peak at 3–5 min activation, while the amount of polymerized vimentin was unchanged (Fig. 3*C*). The distribution of γ-tubulin examined by means of specific Abs was found basically unchanged during activation and was located both on centrosomes and in the cytoplasm (Fig. 1*F*). Collectively, these data demonstrate that early stages of cell activation, when microtubule formation is stimulated, are characterized by tyrosine-phosphorylated proteins concentrating in the centrosomal region of the cell, where γ-tubulin is accumulated.

To determine whether γ-tubulin forms de novo complexes with tyrosine-phosphorylated proteins in activated cells, immunoprecipitation experiments were performed with cell lysates prepared from cells activated by FcεRI aggregation or by pretreatment with pervanadate. Using anti-γ-tubulin Ab TU-31 immobilized on protein A, and cell lysate from nonactivated cells, only small amount of tyrosine-phosphorylated proteins coprecipitated with γ-tubulin. (Fig. 4, *panel* P-Tyr, *lane 1*). However, in cells activated with Ag for 1 min the amounts of coprecipitated and phosphorylated proteins increased and weres further enhanced after another 2 min (Fig. 4, *panel* P-Tyr, *lanes 2* and *3*). The amount of coprecipitated proteins decreased after 6 min activation (data not shown). No staining was seen when protein A alone was incubated with extracts from activated cells (Fig. 4, *panel* P-Tyr, *lane 4*) or when the immobilized Ab was incubated without the extract (Fig. 4, *panel*

FIGURE 3. Immunofluorescence localization of tyrosine-phosphorylated proteins and β-tubulin in resting and activated Lyn$^{-/-}$ BMMCs. *A*, The resting cells (*a–c*) or cells activated for 3 min by FcεRI aggregation (*d–f*) were stained by double labeling with Abs specific for phosphotyrosine (*a* and *d*; green) and β-tubulin (*b* and *e*; red). *c* and *f*, Superpositions of stainings in each row. All photographs were taken under the same exposure conditions. *B*, The resting cells (*a–c*) or cells treated with pervanadate for 3 min (*d–f*) or 15 min (*g–i*) were stained by double labeling with Abs specific for phosphotyrosine (*a*, *d*, and *g*; green) and β-tubulin (*b*, *e*, and *h*; red). *c*, *f*, and *i*, Superpositions of stainings in each row. All photographs were taken under the same exposure conditions. Scale bars, 10 μm. *C*, The resting cells or cells stimulated for various time intervals (1–15 min) by pervanadate were extracted in 0.2% Triton X-100, and detergent-insoluble fractions were analyzed by immunoblotting using Ab against α-tubulin. Anti-vimentin Ab was used as loading control. Numbers under the blot indicate relative amount of α-tubulin normalized to unstimulated control. Means ± SD were calculated from three experiments.
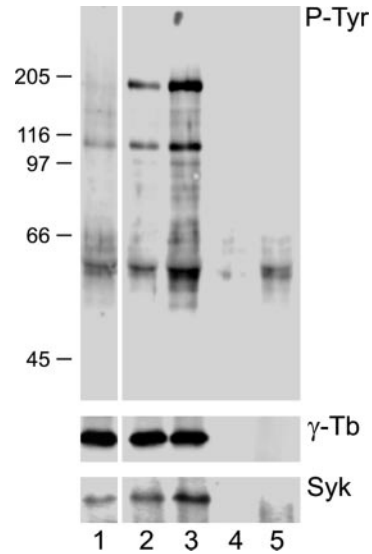
FIGURE 4. γ-Tubulin-associated proteins in resting and FcεRI-activated Lyn$^{-/-}$ BMMCs. γ-Tubulin was precipitated with TU-31 Ab immobilized to protein A beads and the blots were probed with Abs against phosphotyrosine (P-Tyr), γ-tubulin (γ-Tb), and Syk kinase (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells activated by FcεRI aggregation for 1 min (*lane 2*) or 3 min (*lane 3*). Negative control precipitations from activated cells (3 min) using protein A beads without Ab (*lane 4*) or protein A beads with anti-γ-tubulin Ab but without cell extract (*lane 5*). Positions of molecular mass markers (in kilodaltons) are indicated on the *left*. A typical results from four experiments performed.

P-Tyr, *lane 5*). Staining of the precipitated material with anti-γ-tubulin Ab confirmed the presence of γ-tubulin in both unstimulated and stimulated cells (Fig. 4, *panel* γ-Tb, *lanes 1–3*). Similar experiments with pervanadate-activated cells yielded complexes of comparable properties but formation of γ-tubulin assemblies and the extent of tyrosine phosphorylation were more pronounced. From nonactivated cells, only a small amount of tyrosine-phosphorylated proteins coprecipitated with γ-tubulin. (Fig. 5, *panel* P-Tyr, *lane 1*). However, after a 3-min stimulation, the amount of coprecipitated proteins increased and was further enhanced after another 12 min (Fig. 5, *panel* P-Tyr, *lanes 2* and *3*). Close inspection showed that γ-tubulin associated with tyrosine-phosphorylated proteins with relative molecular weights around 50, 60, 70, 80–97, 110, and 200 kDa. Similar staining pattern was observed with different Abs against phosphotyrosine proteins (mAbs 4G10 or PY-20 and polyclonal Ab; data not shown). No such staining was observed with negative controls (Fig. 5, *panel* P-Tyr, *lanes 4* and *5*). When a negative control Ab NF-09 (IgG2a) was used, no phosphotyrosine proteins were detected (data not shown), proving the specificity of the observed reactivity. γ-Tubulin was present in both unstimulated and stimulated cells (Fig. 5, *panel* γ-Tb, *lanes 1–3*). When the immunoprecipitated proteins were probed with polyclonal Ab against α-tubulin, a distinct faint band was detected in control as well as in activated cells in the position of α-tubulin (Fig. 5, *panel* α-Tb, *lanes 1–3*). It is unlikely that the observed associations of the proteins reflect unspecific interactions because a number of other cytoplasmic proteins, including actin, showed no such association. A comparison of tyrosine-phosphorylated proteins associated with γ-tubulin from wild-type with those from Lyn$^{-/-}$ BMMC failed to reveal any substantial qualitative and quantitative differences, except a more intense staining of ~70-kDa protein in wild-type BMMC (data not shown).
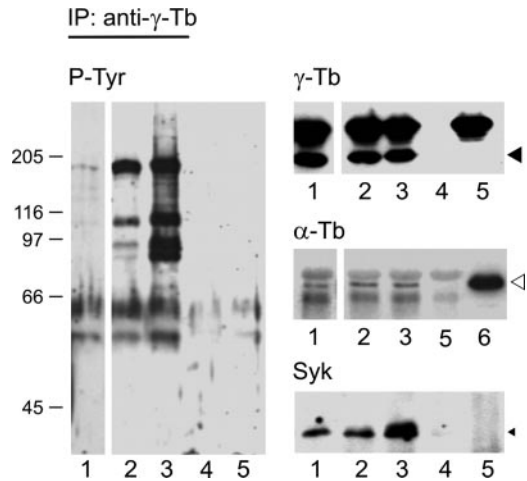
FIGURE 5. γ-Tubulin-associated proteins in resting and pervanadate-activated Lyn$^{-/-}$ BMMCs. γ-Tubulin was precipitated with TU-31 Ab immobilized to protein A beads, and the blots were probed with Abs against phosphotyrosine (P-Tyr), γ-tubulin (γ-Tb), α-tubulin (α-Tb), and Syk kinase (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells treated with pervanadate for 3 min (*lane 2*) or 15 min (*lane 3*). Negative control precipitations from pervanadate activated cells (15 min) using protein A beads without Ab (*lane 4*) or protein A beads with anti-γ-tubulin Ab but without cell extract (*lane 5*). Position of tubulin in cell extract is shown in *lane 6*. Positions of molecular mass markers (in kilodaltons) are indicated on the *left* in panel P-Tyr. Black arrowhead, white arrowhead, and small arrowhead point to γ-tubulin, α-tubulin, and phosphorylated Syk, respectively. A typical result from four experiments performed.

Association of γ-tubulin with kinases

To find out whether γ-tubulin forms complexes with kinases, γ-tubulin was precipitated from nonactivated or FcεRI-activated cells. Activation of the cells was alternatively induced with pervanadate which gave somewhat stonger signal. Data presented in Fig. 4, *panel Syk, lanes 1–3,* clearly show that Syk kinase associates with γ-tubulin in FcεRI-activated cells and that the amount of Syk immunoprecipitated with γ-tubulin correlates with the extent of protein tyrosine phosphorylation. Similar results were observed with pervanadate (Fig. 5, *panel Syk*). Additional experiments showed that γ-tubulin could be precipitated specifically with anti-Fyn-, anti-Syk-, and anti-P-Tyr-specific Abs (Fig. 6, *A–C, lanes 2* and *3*). In precipitates with anti-phosphotyrosine Ab, more γ-tubulin was observed in activated than in resting cells. No staining in the position of γ-tubulin was observed when immobilized Abs were incubated without the extract (Fig. 6, *A–C, lane 1*) or when protein A without the Ab was incubated with extracts from stimulated cells (data not shown). Immunostaining with anti-Fyn Ab confirmed the presence of Fyn kinase in the precipitate (Fig. 6*D*). Labeling with anti-phosphotyrosine Ab showed that a protein phosphorylated on tyrosine was present in a position corresponding to Fyn kinase in both resting and activated cells, however, in activated cells Fyn showed an enhanced phosphorylation (Fig. 6*G*). In precipitates with anti-Syk Ab, nonphosphorylated and phosphorylated forms of Syk kinase were observed in activated cells (Fig. 6*E*). This was confirmed by labeling with anti-phosphotyrosine Ab (Fig. 6*H*). In precipitates with anti-phosphotyrosine Ab, both phosphorylated forms of Syk kinase (Fig. 6*F*) and Fyn kinase (Fig. 6*I*) were detected in enhanced amounts in activated cells. When negative control rabbit Ab against myosin was used for immunoprecipitation of the extract from stimulated cells, no γ-tubulin was detected (data not shown). Basically, the same results were obtained with lysates from wild-type BMMCs (data not shown). To rule out the possi-
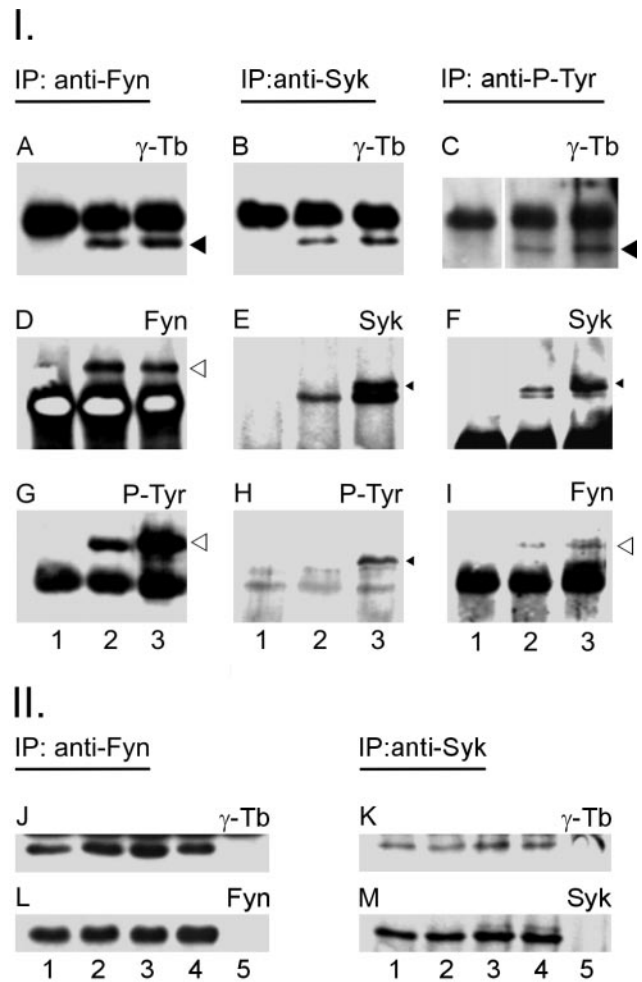
FIGURE 6. Immunoprecipitation of Fyn and Syk from Lyn$^{-/-}$ BMMCs stimulated by pervanadate (*I*) or FcεRI aggregation (*II*). *I*, Cell extracts were precipitated with protein A immobilized Abs specific to Fyn kinase (*A, D,* and *G*), Syk kinase (*B, E,* and *H*), or phosphotyrosine (*C, F,* and *I*). Blots were probed with Abs against γ-tubulin (γ-Tb), kinase Fyn (Fyn), kinase Syk (Syk), or phosphotyrosine (P-Tyr). Immobilized Igs not incubated with cell extract (negative control, *lane 1*), immunoprecipitated proteins from resting cells (*lane 2*), and from cells treated with pervanadate for 15 min (*lane 3*). Black arrowhead, white arrowhead, and small arrowhead point to γ-tubulin, Fyn kinase, and phosphorylated Syk kinase, respectively. *II*, Cell extracts were precipitated with protein A immobilized Abs specific to Fyn kinase (*J* and *L*) and Syk kinase (*K* and *M*). Blots were probed with Abs against γ-tubulin (γ-Tb), kinase Fyn (Fyn), and kinase Syk (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells activated by FcεRI aggregation for 1 min (*lane 2*), 3 min (*lane 3*), or 6 min (*lane 4*). Immobilized Igs not incubated with cell extract (negative control, *lane 5*). A typical result from four experiments performed.

bility that the association of γ-tubulin with kinases is only due to indirect protein associations within large phosphoprotein aggregates, precipitation experiments with anti-Fyn and anti-Syk Abs were also performed with FcεRI-activated cells. In cells activated with Ag, the amount of γ-tubulin coprecipitated with anti-Fyn Ab increased with a peak 3 min after triggering (Fig. 6*J, panel γ-Tb, lanes 1–4*). Similarly, a peak in the amount of γ-tubulin coprecipitated with Syk was observed at 3 min in FcεRI-activated cells (Fig. 6*K, panel γ-Tb, lanes 1–4*). The combined data indicate that soluble γ-tubulin in activated cells appears in complexes with Fyn and Syk kinases and several other proteins phosphorylated on tyrosine. Importantly, the formation of these complexes is not dependent on the presence of Lyn kinase.

Binding of γ-tubulin complexes to the regulatory domains of Fyn kinase

Guided by our finding that γ-tubulin in Lyn$^{-/-}$ BMMCs forms complexes with Fyn kinase, we further investigated whether the SH2 and/or SH3 domains of Fyn participate in these interactions. Data presented in Fig. 7 show that γ-tubulin complex binds to GST-Fyn-SH2 as well as GST-Fyn-SH3 fusion proteins, but not to GST alone. Under identical conditions, more γ-tubulin was bound to GST-Fyn-SH2 than to GST-Fyn-SH3. In activated cells, more γ-tubulin complex was bound to GST-Fyn SH2, but there was no difference in binding of γ-tubulin complex from resting and activated cells to the GST-Fyn-SH3 (Fig. 7A, *panel* γ-Tb). The same distribution pattern of γ-tubulin was observed with anti-γ-tubulin Abs GTU-88 and TU-32 that are directed against different epitopes on γ-tubulin molecule (data not shown). The amount of immobilized GST fusion proteins was similar as detected by staining with anti-GST Ab (Fig. 7A, *panel* GST). Using two different Abs against α-tubulin (DM1A and TU-01) and two different Abs against β-tubulin (TUB 2.1 and TU-06), we failed to detect the binding of αβ-tubulin dimers to GST-fusion proteins, and the same holds true for negative-control anti-actin Ab (data not shown). Interestingly, strong binding of phosphorylated Syk kinase to GST-Fyn-SH2 was observed in activated cells (Fig. 7B, *panels* Syk); however, no binding of Syk to GST-Fyn-SH3 was detected under identical conditions (data not shown). More γ-tubulin was also found in GST-Fyn-SH2 pull-down complexes when lysates from FcεRI-activated cells were used (data not shown).

To determine whether the observed interactions of γ-tubulin complex with the SH2 domain of Fyn kinase reflect an SH2-phosphotyrosine type interaction, we performed competition experiments with extracts from activated Lyn$^{-/-}$ BMMCs and phenyl phosphate, an analog of phosphotyrosine. Phenyl phosphate inhibited in a concentration-dependent manner the binding of γ-tubulin complex to GST-Fyn-SH2; IC$_{50}$ was attained at 7.5 mM phenyl phosphate. This inhibition was specific, because phosphoserine had no effect on the binding (data not shown). Similarly, the binding of Syk to GST-Fyn-SH2 was inhibited by phenyl phosphate (IC$_{50}$ at 9 mM), while phosphoserine (up to 40 mM) was without effect. The relatively high concentration of phenyl phosphate required to get IC$_{50}$ implies that the inhibitor has low specificity. Additional experiments, therefore, made use of the peptide containing the most preferable motif (pY-E-E-I) for binding to Fyn-SH2 domain (47). The results showed that the phosphorylated peptide (PQpYEEIPI) reduced the binding of γ-tubulin complex to GST-Fyn-SH2 domain (Fig. 7B, *panel* γ-Tb). The IC$_{50}$ was attained at the concentration of 14 μM (Fig. 7C). The unphosphorylated form of the same peptide was without effect. The phosphorylated oligopeptide also inhibited the binding of Syk to GST-Fyn-SH2 (Fig. 7B, *panel* Syk) with IC$_{50}$ attained at 21 μM. These data suggest that the bindings were mediated by an interaction of Fyn-SH2 domain with tyrosine-phosphorylated residues present in adaptor proteins associated with the complexes. Experiments repeated with FcεRI-activated cells gave similar results (data not shown). Treatment of the cells with Src family specific inhibitor SU6656 before activation resulted in a lower amount of γ-tubulin and phosphorylated Syk associated with GST-Fyn-SH2 as revealed by pull-down experiments (Fig. 8). Another Src family inhibitor PP2 had a similar effect (data not shown).

To decide whether the observed interactions of γ-tubulin with Fyn-SH2 and Fyn-SH3 domains are direct or indirect, we searched the ubiquitously expressed (48) mouse γ-tubulin (Swiss-prot accession no. P83887) for consensus sequences that could be involved directly in binding to Fyn SH2 or SH3 domains. The data
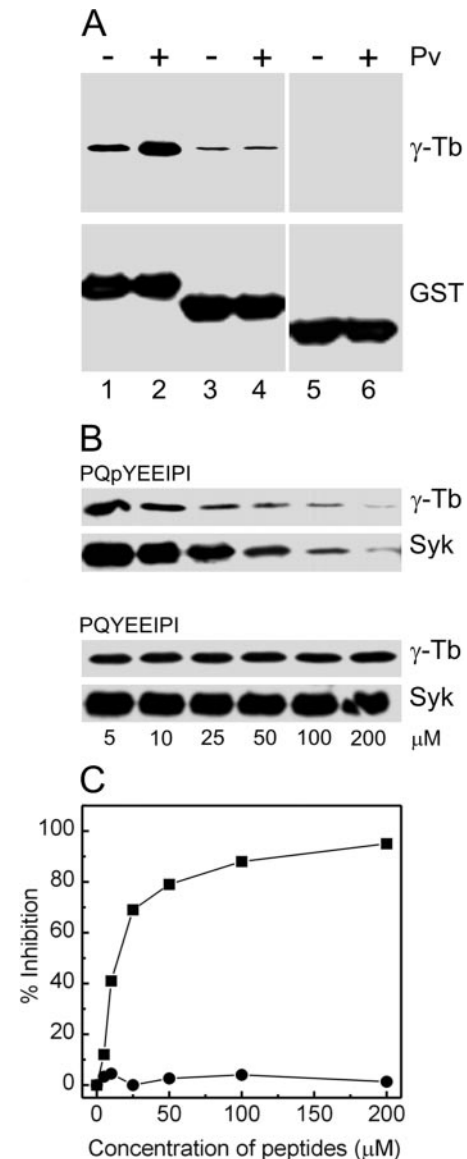
FIGURE 7. Binding of γ-tubulin and Syk kinase to SH2 or SH3 domains of Fyn kinase. *A,* Postnuclear supernatants from resting (−) or pervanadate-activated cells (+; 15 min) were incubated with GST fusion proteins or GST alone (negative control) immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Blots were probed with Ab against γ-tubulin (γ-Tb) or Ab against GST. GST-SH2 domain of Fyn kinase (*lanes 1* and *2*), GST-SH3 domain of Fyn kinase (*lanes 3* and *4*), GST alone (*lanes 5* and *6*). *B,* Effect of tyrosine-phosphorylated oligopeptide (PQpYEEIPI) and its nonphosphorylated form (PQYEEIPI) on binding of γ-tubulin (γ-Tb) and Syk kinase (Syk) to SH2 domain of Fyn kinase. GST-SH2 domain of Fyn kinase was incubated with cell extract from pervanadate-activated cells in the absence (0 mM) or presence of tyrosine-phosphorylated oligopeptide (PQpYEEIPI) and its nonphosphorylated form at concentrations 5–200 μM. *C,* Quantification of data for γ-tubulin presented in (*B*). Extent of binding of γ-tubulin complexes to the GST-SH2 domain of Fyn kinase in the presence of PQpYEEIPI oligopeptide (■—■) or PQYEEIPI oligopeptide (●—●) was determined by densitometric analysis.

show that γ-tubulin possesses 15 tyrosine residues, but none of them is within the most preferable motif (Y-E-E-I) for binding to Fyn-SH2 domain. However, one tyrosine (residue 186) fits to the general amino acid consensus sequence (Y-hydrophilic-hydrophilic-hydrophobic) recognized by SH2 domains of the Src family kinases (47). Moreover, γ-tubulin contains two consensus motifs
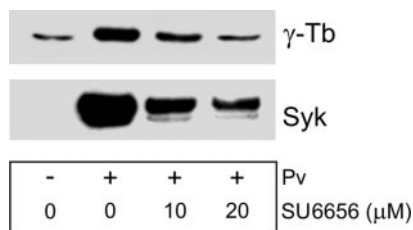
FIGURE 8. Effect of Src-family specific inhibitor on the binding of γ-tubulin or Syk kinase to the SH2 domain of Fyn kinase. Lyn$^{-/-}$ BMMCs were incubated with SU6656 inhibitor at final concentration 10 and 20 μM or without inhibitor and then activated (+) or not (−) with pervanadate (15 min). Postnuclear supernatants were incubated with GST-Fyn-SH2 fusion proteins immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Blots were probed with Ab against γ-tubulin (γ-Tb) or Ab against Syk kinase (Syk).

for binding to SH3 domains, P-X-X-P (49). In further competition experiments, we therefore used the oligopeptides FIPWGPAS covering the sequence 348–355 of γ-tubulin and KSPYLPSA covering the sequence 363–370. The P-X-X-P sequences are underlined. However, even at the highest concentration of the tested peptides used (5 mM) there was no inhibition of binding to Fyn SH3 domain. We also used the oligopeptide PYNSLL, covering the sequence 185–190 of mouse γ-tubulin and its tyrosine-phosphorylated form, in competition experiments with the SH2 domain of Fyn. Unphosphorylated oligopeptide was without effect and its phosphorylated form gave only 30% inhibition at a concentration 3 mM (data not shown), indicating a low specificity of inhibition reflecting just the presence of phosphotyrosine in the peptide. Collectively, these data suggest that the binding of γ-tubulin to SH2 and SH3 domains of Fyn kinase is indirect.

Phosphorylation of tubulin dimers by protein tyrosine kinases associated with γ-tubulin complexes

Possible associations of γ-tubulin with kinases and their substrates were also examined by immunocomplex kinase assays. Lysates from resting Lyn$^{-/-}$ BMMCs or cells stimulated with pervanadate or FcεRI aggregation were precipitated with anti-γ-tubulin Ab (TU-31) or a negative control Ab NF-09. Immunocomplexes were then subjected to the in vitro kinase assays and analyzed by SDS-PAGE followed by electroblotting and autoradiography. γ-Tubulin in the extract from resting cells was associated with several kinase substrates ranging from 40 to 200 kDa (Fig. 9A, lane 1). When activated, the pattern of labeled proteins was similar, but the amount of ^{32}P-labeled proteins increased (Fig. 9A, lanes 2 and 3). No kinase activity was detected after precipitation with the control Ab (Fig. 9A, lane 4). The labeling patterns using lysate from resting and pervanadate-stimulated (15 min) wild-type BMMCs are shown in Fig. 9A (lanes 5–6). Compared with Lyn$^{-/-}$ BMMCs, stronger signals were detected, namely at a region of ∼70 kDa. The staining of size-separated ^{32}P-labeled proteins in γ-tubulin immunocomplexes, from Lyn$^{-/-}$ BMMCs, subjected to the in vitro kinase assay with anti-phosphotyrosine Ab revealed that the dominant ^{32}P-labeled proteins of 50, 80–97, 110, and 200 kDa contained phosphotyrosine. Staining with Ab against phospho-serine showed only a faint staining of 110- and 200-kDa proteins. In contrast, no staining was detected with Ab specific for phos-phothreonine (data not shown).

When cells before activation were cultured in the presence of Src family selective tyrosine kinase inhibitor PP2, a lower level of phosphorylation was detected (Fig. 9B, lane 2). In contrast, the presence of PP3 (negative control to PP2) had no effect (data not

shown). Similarly, when the cells were cultured in the presence of Syk selective tyrosine kinase inhibitor piceatannol (dissolved in DMSO), a clear inhibition of phosphorylation was detected (Fig. 9C, lane 2). No inhibition was observed when DMSO alone was used. Staining of parallel blots with anti-phosphotyrosine and anti-Syk Abs revealed that PP2 pretreatment inhibited the phosphory-lation of Syk kinase (data not shown).

Because γ-tubulin forms a complex with tubulin dimers (Fig. 5), we wanted to find out whether tubulin dimers could serve as a substrate for kinases present in the γ-tubulin immunocomplexes. When exogenous αβ-tubulin dimers (5 μg) were added to γ-tu-bulin immunocomplexes, phosphorylation of this extra tubulin was observed in kinase assay (Fig. 9D, lane 3). In cells pretreated with PP2, the extent of tubulin labeling was lower (Fig. 9D, lane 4). The results of similar experiments with extracts from cells pretreated with piceatannol also showed lower phosphorylation of exogenous tubulin (Fig. 9E, lanes 3 and 4). When BSA (5 μg) was added to kinase mixture no labeling of this protein was observed. These data demonstrate that both active Src family kinase(s) and active Syk kinase are part of the γ-tubulin complexes in Lyn$^{-/-}$ BMMCs and that these kinases can be involved in phosphorylation of tubulin dimers.

To prove that an enhanced kinase activity in γ-tubulin com-plexes is also present in cells activated by FcεRI aggregation, the cells were stimulated with Ag and the kinase activity of γ-tubulin immunocomplexes was evaluated by kinase assay and autoradiog-raphy. Enhanced kinase activity in γ-tubulin immunocomplexes detected in FcεRI-activated cells (Fig. 9F) proved that an associ-ation of γ-tubulin with kinases also occurred also under more physiological conditions (Fig. 9F). When the cells were exposed to Src kinase inhibitor PP2 (20 μM) and then activated by FcεRI aggregation (3 min), a lower level of phosphorylation of proteins associated with γ-tubulin immunocomplexes was detected (data not shown).

Discussion

FcεRI aggregation in mast cells and basophiles leads to rapid cy-toskeleton rearrangements that are important for cell activation and degranulation. Both actin filaments and microtubules play a critical role in this process (7, 8, 10, 50, 51). Data presented in this study show that stimulation of mast cells through FcεRI aggregation or pervanadate exposure triggers the formation of microtubules in both wild-type and Lyn$^{-/-}$ BMMCs, as documented by immuno-fluorescence microscopy. In our previous study, we have found that Lyn kinase, a major Src family kinase in RBL-2H3 cells (52), forms complexes containing γ-tubulin and phosphotyrosine pro-teins, and we proposed that Lyn might be involved in microtubule formation (29). To shed more light on the role of Lyn kinase in the formation of γ-tubulin-based complexes, we have primarily ana-lyzed the properties of γ-tubulin immunocomplexes isolated from Lyn-deficient BMMCs. Wild-type BMMCs served as controls. The first evidence that Lyn kinase is dispensable for the formation of functional γ-tubulin complexes was our finding of normal to-pography of microtubules in Lyn$^{-/-}$ BMMCs. Importantly, FcεRI-induced cell activation resulted in an enhanced microtubule formation, and no difference was observed between wild-type and Lyn$^{-/-}$ BMMCs. More intense formation of microtubules was observed in pervanadate-stimulated cells, supporting the concept that an enhanced activity of kinases and/or shift in balance be-tween kinases and phosphatases is required to accomplish this process.

Several lines of evidence indicated that γ-tubulin extracted from mast cells with nonionic detergent Nonidet P-40-formed com-plexes with signal transduction molecules that could modulate the
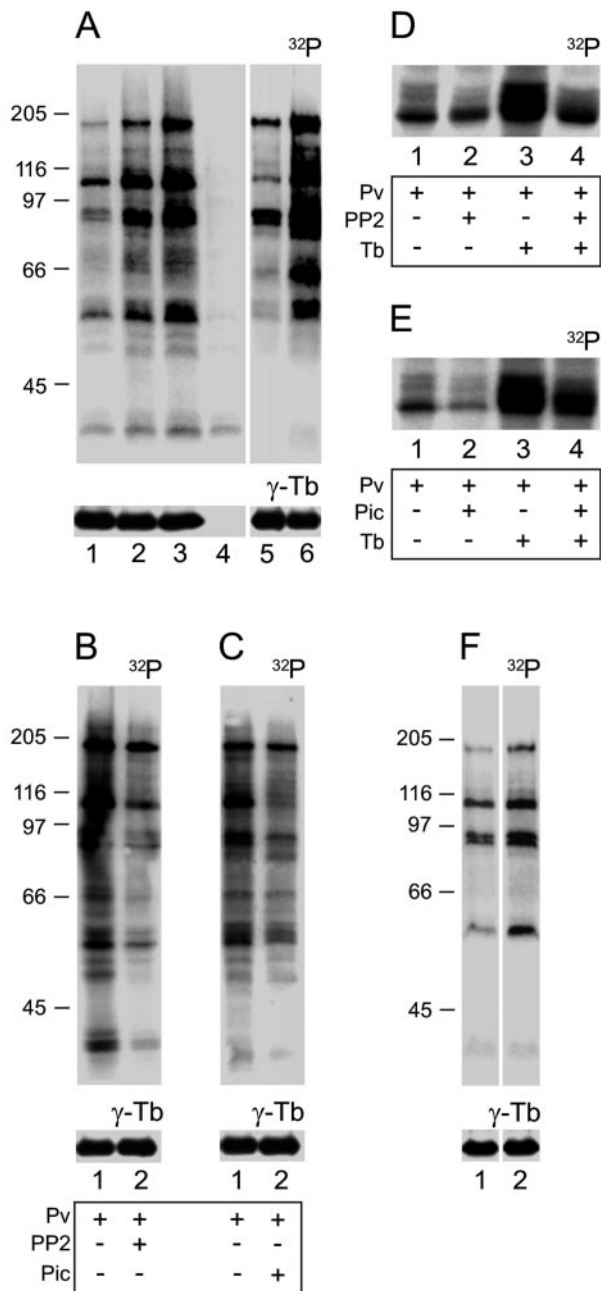
FIGURE 9. The kinase activity in γ-tubulin immunocomplexes from Lyn^{−/−} BMMCs. Samples were prepared from pervanadate-activated (*A* and *E*) or from FcεRI-activated (*F*) cells. Cell lysates were precipitated with anti-γ-tubulin Ab TU-31 or negative control Ab NF-09 bound to immobilized protein A. Immunocomplexes were subjected to in vitro kinase assay, electrophoretically separated, and detected by autoradiography (^{32}P). The presence of γ-tubulin in immunocomplexes was confirmed by immunoblotting with anti-γ-tubulin Ab (γ-Tb). *A*, Precipitation from resting cells (*lane 1*), cells treated with pervanadate for 3 min (*lane 2*) or 15 min (*lane 3*). Precipitation with negative control Ab NF-09 from 15 min pervanadate-treated cells (*lane 4*). For comparison, the kinase activity after precipitation from resting (*lane 5*) and pervanadate-activated (15 min) wild-type BMMCs (*lane 6*) is shown. *B*, Comparison of kinase activity from pervanadate-activated cells pretreated with DMSO alone (*lane 1*, control) or Src kinase family selective inhibitor PP2 (20 μM) before activation (*lane 2*). *C*, Comparison of kinase activity from pervanadate-activated cells pretreated with DMSO alone (*lane 1*) or Syk kinase inhibitor piceatannol (Pic, 50 μM) (*lane 2*). *D*, Kinase activity from pervanadate-activated cells without (*lanes 1* and *3*) or with PP2 pretreatment (*lanes 2* and *4*). Exogenous αβ-tubulin dimers were added to immunocomplexes before kinase assay (*lanes 3* and *4*). *E*, Kinase activity from pervanadate-activated cells

microtubule arrays. First, tyrosine-phosphorylated proteins were found to be associated with immunoprecipitated γ-tubulin in resting cells and the amount of these proteins increased after activation. Second, in vitro kinase assays revealed that γ-tubulin formed complexes containing kinases and their substrates. Increased ^{32}P labeling of proteins was observed when kinase assays were performed with lysates from cells exposed to pervanadate or cells activated by FcεRI aggregation. Third, the activity of γ-tubulin-associated kinases was inhibited by pretreatment of the cells with Src-family specific (PP2) and Syk-specific (piceatannol) inhibitors; this suggests that Src and Syk family kinases have an important role in the formation of γ-tubulin-signaling complexes. Fourth, association of Fyn kinase with γ-tubulin complexes was confirmed by immunoprecipitation experiments, and the same holds true for Syk kinase. Finally, kinases in γ-tubulin complexes were capable of using tubulin dimer as a substrate, and its phosphorylation was inhibited by both PP2 and piceatannol. Whether other kinases become associated with γ-tubulin complexes in the course of cell activation remains to be determined.

Interaction of γ-tubulin complexes with Fyn was confirmed by pull-down experiments, in which γ-tubulin complex bound to SH2 domain of Fyn kinase in a phosphotyrosine-dependent manner. Similar binding has already been described in embryonal carcinoma P19 cells during neuronal differentiation (28). However, in contrast to P19 cells, Fyn from Lyn^{−/−} BMMC bound to γ-tubulin complexes not only through the SH2 domain, but also the SH3 domain. Although γ-tubulin possesses two potential binding sites for SH3 domains and one for SH2 domain, experiments with phenyl phosphate and synthetic peptide inhibitors failed to confirm a direct binding of γ-tubulin to these domains. Moreover, when purified GST-Fyn-SH2 and GST-Fyn-SH3 fusion constructs were used to probe size-fractionated proteins from activated Lyn^{−/−} BMMCs on nitrocellulose membranes, no specific staining was detected with anti-GST Ab in the γ-tubulin region. Thus, the association with the SH2 domain is probably mediated via adaptor-like tyrosine-phosphorylated protein(s). The binding of γ-tubulin complex with the SH2 domain-containing proteins is probably not mediated by tyrosine-phosphorylated tubulin dimers as we were unable to detect them in pull-down experiments with Fyn SH2 domains using various anti-tubulin Abs. Yet, a binding of α-tubulin to SH2 domain of Fyn was observed in activated human T lymphocytes (53). Either different cell type used or binding of very low amount of tubulin dimer, under the detection limit of our assay, might account for the discrepancy. Concerning γ-tubulin immunocomplexes, further studies are in progress to elucidate their composition, mode of interaction and functions of individual components in mast cell signaling. Fyn kinase, like other Src family kinases, is commonly involved in the formation of multiprotein complexes engaged in interaction with the SH2 and SH3 domains. It is therefore likely that the association of Fyn with γ-tubulin is mediated through other proteins that are also important for microtubule nucleation. Currently, we are verifying whether some of the phosphotyrosine proteins of γ-tubulin immunocomplexes in activated cells belong to the class of proteins of the large γ-TuRC.

The molecular mechanism of the association of Syk kinase with γ-tubulin is unclear. Our study shows that phosphorylated Syk is among the proteins bound to the SH2 domain of Fyn kinase. This

without (*lanes 1* and *3*) or with piceatannol pretreatment (*lanes 2* and *4*). Exogenous αβ-tubulin dimers were added to immunocomplexes (*lanes 3* and *4*). *F*, Precipitation from resting cells (*lane 1*) and from cells stimulated by FcεRI aggregation for 3 min (*lane 2*). Molecular mass markers (in kilodaltons) are indicated on the *left* in *A*, *B*, and *F*.

suggests a direct binding of Fyn-SH2 with the phosphotyrosine of Syk. Direct binding of phosphorylated Syk to the SH2 domain of Lyn kinase was described in RBL-2H3 cells (5). Syk has one general amino acid consensus sequence that is recognized by SH2 domains of Src family kinases (5). In RBL as well as in BMMCs, Syk is one of the preferable substrates for Lyn kinase (54). However, even in Lyn$^{-/-}$ BMMCs there is still some phosphorylation of Syk on tyrosine, which is dependent on FcεRI activation (4). Because PP2 inhibited phosphorylation of Syk in pervanadate-activated Lyn$^{-/-}$ BMMCs and phosphorylated Syk bound to GST-Fyn-SH2 in a phosphotyrosine-dependent manner, it is likely that Fyn participates in the phosphorylation of Syk in these cells. These data, together with our finding that piceatannol diminished phosphorylation of proteins in γ-tubulin immunocomplexes, suggest that it is the cross-talk between Fyn and Syk which is responsible for tyrosine phosphorylation of proteins associated with γ-tubulin immunocomplexes in Lyn$^{-/-}$ BMMCs. Whether Fyn can directly phosphorylate Syk in FcεRI-activated Lyn$^{-/-}$ BMMCs remains to be elucidated. Association of Lyn kinase with γ-tubulin was described in RBL cells (29). One might speculate that Fyn kinase could substitute for Lyn in Lyn$^{-/-}$ BMMCs in phosphorylation of those proteins that are important for microtubule nucleation in the course of the activation process. An important regulator of γ-tubulin functions could be the Syk kinase. In wild-type cells Syk kinase is phosphorylated predominantly by Lyn kinase, whereas in Lyn$^{-/-}$ BMMCs, the Fyn kinase could assume this role.

Tubulin has been shown to serve as a substrate for Syk kinase in vivo (55). Syk can phosphorylate both soluble tubulin (56) and tubulin in microtubules (57). Syk phosphorylates α-tubulin on the conserved tyrosine residue (Tyr^{432}) and Syk-selective inhibitor blocks receptor-stimulated tubulin phosphorylation in B lymphocytes (55). However, phosphorylation of tubulin by Syk did not have any profound effect on microtubule assembly in pervanadate-treated cells (57). Furthermore, phosphorylation of tubulin by Src kinase did not cause any significant changes in microtubule polymer (58). It is, therefore, unlikely that phosphorylation of tubulin dimers plays a key role in the increase of microtubule formation.

The formation of microtubules can be regulated either by stabilizing the plus ends of microtubules or by regulating the microtubule nucleation where γ-tubulin plays a key role. It has been shown that the Fyn/Gab2/RhoA-signaling pathway plays a critical role in microtubule-dependent degranulation of mast cells, and that RhoA kinase could be involved in stabilization of the plus ends of microtubules (10). Our data suggest that Fyn also might be involved in phosphorylation of proteins that regulate γ-tubulin interactions. As we saw no evidence of Gab2 association with γ-tubulin immunocomplexes (V. Sulimenko, unpublished data), it seems unlikely that γ-tubulin directly participates in the Fyn/Gab2/RhoA-signaling pathway. However, as we and others have found, γ-tubulin is phosphorylated (27, 28, 59). Phosphorylation of the γ-tubulin residue Tyr^{445}, which is invariably present in all γ-tubulins, was described in budding yeast; a mutation of this residue changed the microtubule dynamics (27). There are other data that point to an association of γ-tubulin with kinases. PI3K binds to γ-tubulin in response to insulin (25), and the 55-kDa regulatory subunit of PI3K interacts with γ-tubulin (26). Besides, we have found that Src family kinases appear in complexes with γ-tubulin in RBL cells (29). Collectively, these data suggest that kinases take part in the regulation of γ-tubulin function(s). This could lead to changes in nucleation properties of centrosomes or alternatively to an enhancement of noncentrosomal microtubule nucleation.

Our data show that both Fyn kinase and γ-tubulin are associated in detergent-resistant fraction of BMMCs. Interestingly, there are several reports pointing to the localization of Fyn and Syk kinases to centrosomal region. Fyn kinase was localized in different cell types in centrosomes and in microtubule bundles radiating from the centrosome (53, 60, 61). Furthermore, Fyn kinase was also found to be associated with microtubules of meiotic cells (62). Finally, Syk was located at the centrosomes in B lymphocytes (63). Thus, tyrosine phosphorylation of centrosomal proteins by Fyn and Syk kinases could be the process linking microtubules to early activation events in BMMCs.

In conclusion, it appears that activation of BMMCs leads to a rapid formation of microtubules and that γ-tubulin forms complexes with Fyn and Syk kinases and other signal transduction molecules in the process of cell activation. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins and thus modulate the microtubule nucleation in activated mast cells.

Acknowledgments

Disclosures

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