ELSEVIER

Biochimica et Biophysica Acta 1524 (2000) 17–26

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Bacterial peptidoglycan binds to tubulin

Roman Dziarski [a,*], Mark M. Rasenick [b], Dipika Gupta [a]

[a] *Northwest Center for Medical Education, Indiana University School of Medicine, 3400 Broadway, Gary, IN 46408, USA*
[b] *Department of Physiology and Biophysics, University of Illinois, College of Medicine, Chicago, IL 60612, USA*

Received 21 June 2000; received in revised form 29 August 2000; accepted 31 August 2000

Abstract

A search for cellular binding proteins for peptidoglycan (PGN), a CD14- and TLR2-dependent macrophage activator from Gram-positive bacteria, using PGN-affinity chromatography and N-terminal micro-sequencing, revealed that tubulin was a major PGN-binding protein in mouse macrophages. Tubulin also co-eluted with PGN from anti-PGN vancomycin affinity column and bound to PGN coupled to agarose. Tubulin-PGN binding was preferential under the conditions that promote tubulin polymerization, required macromolecular PGN, was competitively inhibited by soluble PGN and tubulin, did not require microtubule-associated proteins, and had an affinity of 100–150 nM. By contrast, binding of tubulin to lipopolysaccharide (LPS) had 2–3 times lower affinity, faster kinetics of binding, and showed positive cooperativity. PGN enhanced tubulin polymerization in the presence of 4 M glycerol, but in the absence of glycerol, both PGN and LPS decreased microtubule polymerization. These results indicate that tubulin is a major PGN-binding protein and that PGN modulates tubulin polymerization. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lipopolysaccharide; Microtubule; Peptidoglycan; Tubulin

1. Introduction

Peptidoglycan (PGN) is present in the cell walls of all bacteria and is the major constituent of the cell walls of Gram-positive bacteria. PGN can reproduce most of the major clinical manifestations of bacterial infections, including fever, inflammation, hypotension, leukocytosis, sleepiness, decreased appetite, malaise, and arthritis [1–3]. These clinical manifestations are induced by PGN indirectly, through the activation of macrophages and stimulation of secretion of mediators of inflammation, primarily cytokines and chemokines [1–3].

The first step in macrophage activation by PGN is the binding of PGN to its specific receptor, membrane CD14 [2–5]. CD14 also serves as the receptor for other bacterial macrophage activators, including lipopolysaccharide (LPS or endotoxin) from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria, lipoproteins from spirochetes, lipoarabinomannan from mycobacteria, and others [2,3]. The second step in macrophage activation is transmission of the activating signal from the receptor into the cell. However, CD14 is a glycosylphosphatidylinositol-linked rather than a transmembrane molecule and by itself CD14 cannot transmit an activating signal into the cell through the membrane. Therefore, Toll-like receptors, TLR2 and TLR4, serve as the cell-activating co-receptors for Gram-positive bacteria and PGN [6,7], and Gram-negative bacteria and LPS [8–11], respectively. TLRs, however, do not seem to bind these bacterial products with sufficient affinity that could account for cell activation ([6,12]; R. Dziarski, C.J. Kirschning, unpublished). Therefore, TLRs appear to function as signal-transducing rather than bacterial cell wall-binding molecules [12].

While looking for other molecules in mammalian cells that bind bacterial PGN, we discovered that PGN binds to tubulin, which we report in this paper. Tubulin is a heterodimer consisting of α and β chains, which forms microtubules and is present in all eucaryotic cells [13]. Polymerization of tubulin into microtubules requires GTP, and in the cell, most microtubules undergo continuous rapid assembly and disassembly. Microtubules participate in morphogenesis, cell division, and transport of vesicles and or-

Abbreviations: PGN, peptidoglycan; HBSS, Hank's balanced salt solution; LPS, lipopolysaccharide; ReLPS, LPS from *Salmonella minnesota* Re mutant 595; MDP, muramyl dipeptide (MurNAc-L-Ala-D-isoglutamine); MAP, microtubule-associated proteins; sPGN, soluble PGN; TLR, Toll-like receptor

* Corresponding author. Fax: +1-219-980-6566;
E-mail: rdziar@iunhaw1.iun.indiana.edu

0304-4165/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0304-4165(00)00137-9

ganelles [13]. Numerous signal transduction molecules are also associated with microtubules, including mitogen-activated protein kinases [14,15] and G proteins [16,17]. Tubulin, therefore, may also participate in signal transduction events during cell activation.

Interest in the interaction of bacterial LPS with tubulin started when microtubule-stabilizing agent, taxol, was found to mimic LPS-induced activation of macrophages, and when this effect was not observed in LPS-hyporesponsive C3H/HeJ mice [18–20]. Moreover, the genes controlling the responsiveness to taxol and LPS are closely linked [18], and LPS antagonists block the responsiveness to taxol [21,22]. LPS also binds to tubulin [23,24] and microtubules [24], and all LPS-stimulated mitogen-activated protein kinases are associated with microtubules [15]. Furthermore, during cell activation, LPS is rapidly internalized and transported to the perinuclear Golgi apparatus [25,26]. On route to the Golgi complex or after reaching it, LPS could either intercalate into vesicle membranes or leave the vesicles for the cytoplasm [26], which would give it an opportunity to interact with microtubules. Since both PGN and LPS activate cells through the same CD14 and TLR receptors, we hypothesized that both PGN and LPS may interect with tubulin during cell activation and intracellular transport.

Therefore, the objectives of this study were to: (i) determine if PGN binds to tubulin, (ii) characterize PGN binding to tubulin in detail, (iii) compare PGN and LPS binding to tubulin, including determining the affinity of binding, and the requirement for tubulin polymerization and for microtubule-associated proteins, and (iv) determine the effects of PGN and LPS on tubulin polymerization in vitro.

2. Materials and methods

2.1. Materials

Tubulin containing microtubule-associated proteins (MAP) was purified from sheep brains by two cycles of assembly and disassembly [17], and tubulin free of MAP was purified from tubulin by phosphocellulose chromatography [17]. For some experiments (co-elution from vancomycin affinity columns, see below), tubulin was purified from bovine brain by temperature-dependent assembly-disassembly cycles [27] and contained 15% MAP (obtained from Sigma, St. Louis, MO).

Soluble PGN (sPGN) from *Staphylococcus aureus*, a polymeric non-cross-linked PGN of approximate average $M_r = 125\,000$, was purified by vancomycin affinity chromatography [28]. For some experiments, sPGN was biosynthetically labeled with [^{14}C]alanine [28]. Insoluble PGN was isolated from the cell walls of *S. aureus* 845 and sonicated [28]. By quantitative chemical analysis, both sPGN and insoluble PGN had amino acid and amino sugar composition characteristic of *S. aureus* PGN [28], and contained < 24 pg endotoxin mg^{-1} determined by the *Limulus* lysate assay [28,29]. For some experiments, sPGN was digested with lysostaphin [5].

Synthetic analogs of PGN fragments, PGN pentapeptide (L-Ala-D-isoglutaminyl-L-Lys-D-Ala-D-Ala) and muramyl dipeptide (MurNAc-L-Ala-D-isoglutamine, MDP) were from Sigma, and a disaccharide-dipeptide (GlcNAc-β1-4-MDP) was from Calbiochem (La Jolla, CA). No significant endotoxin contamination of these preparations was detected ($\leq$1 ng endotoxin mg^{-1}) [5].

LPS from *Salmonella minnesota* Re 595 (ReLPS, $M_r = 2000$–3000), a mutant devoid of *O*-polysaccharide and most of the core-polysaccharide, is a minimal naturally occurring endotoxic structure of LPS. It was obtained by phenol–chloroform–petroleum ether extraction by Sigma, and its purity was analyzed as before [5]. Purified smooth (complete) LPS, obtained from *Escherichia coli* O113 by phenol-water extraction (refined endotoxin standard, approximate average $M_r = 15\,000$) was obtained from Ribi Immunochem Research (Hamilton, MT). Taxol (paclitaxel from *Taxus brevifolia*) and all other chemicals were obtained from Sigma, unless otherwise indicated.

2.2. Cells, cell lysates, and cell membranes

For the cell activation assays, mouse macrophage RAW264.7 cells were cultured in DMEM medium with 10% fetal calf serum [4]. For obtaining cell lysates and cell membranes for the binding assays, RAW264.7 cells adapted to growth in serum-free HL-1 medium [30] were used, to avoid contamination with potential PGN-binding proteins from serum. To obtain cell lysates, 2×10^9 cells were lysed at 20°C in 40 ml of Hanks Balanced Salt Solution (HBSS) with 1.2% octylglucoside, 5% glycerol, 1 mM Hepes, 230 μg ml^{-1} PMSF, and 25 μg ml^{-1} aprotinin, and undissolved cell fragments were removed by 30 min centrifugation at $3000\times g$, 20°C. To obtain cell membranes, 3.5×10^9 cells were suspended at 4°C in 60 ml of 25 mM Tris–HCl buffer (pH 7.4) with 5 mM $MgCl_2$, 1 mM EGTA, 230 μg ml^{-1} PMSF, and 25 μg ml^{-1} aprotinin, and homogenized for 20 s in a Polytrone (Brinkman Instruments, Westburg, NY) homogenizer on ice, until 75–85% of cells were broken with no visible damage to nuclei [31]. Unbroken cells, nuclei, and debris were removed by 10 min centrifugation at $500\times g$ at 4°C, and the membranes were sedimented by 60 min centrifugation at $240\,000\times g$ at 4°C [31]. Membranes were suspended by sonication on ice in 0.2 M phosphate buffer (pH 7.4) with 5% glycerol, 425 μg ml^{-1} PMSF, and 45 μg ml^{-1} aprotinin, solubilized in 1% octylglucoside, and diluted to 80 ml with HBSS with 0.1% octylglucoside and 1% glycerol. Undissolved membrane aggregates were removed by centrifugation at $25\,000\times g$ at 4°C.

2.3. PGN-affinity chromatography and N-terminal sequencing

In the first approach, cell lysate (40 ml) was diluted with 200 ml of HBSS with 0.2% octylglucoside and 1% glycerol, mixed with 4 ml of sPGN coupled to agarose [5] for 18 h at 4°C, and poured into a column. The sPGN–agarose was washed with 200 ml of HBSS with 0.2% octylglucoside and 1% glycerol, and agarose-bound proteins were eluted with 9 M urea with 2% NP40 and 5 mg ml^{-1} DTT at 70°C, precipitated with 90% ethanol at −20°C for 18 h, sedimented by centrifugation at $3000\times g$, and dissolved in polyacrylamide gel electrophoresis (PAGE) sample buffer with 1 N NaCl, 2% sodium dodecyl sulfate (SDS), and 1% 2-mercaptoethanol. In the second approach, cell lysates diluted as above were centrifuged at $4000\times g$ for 30 min, the supernatants were mixed with 4 mg insoluble PGN for 24 h at 4°C, the PGN was sedimented by centrifugation at $3000\times g$ for 20 min at 4°C, the sediment was washed with HBSS with 0.2% octylglucoside and 1% glycerol, and PGN-bound proteins were eluted with 9 M urea with 2% NP40 and DTT, precipitated with ethanol, and dissolved in PAGE buffer as described above. In the third approach, dissolved membranes were mixed with 1.2 mg insoluble PGN for 6 h at 4°C, PGN was sedimented at $4000\times g$, washed with HBSS with 0.2% octylglucoside and 1% glycerol, and PGN-bound proteins were eluted and dissolved as in the second approach.

Eluted PGN-binding proteins were subjected to SDS–PAGE on 11% gels, blotted onto a PVDF Immobilon P (Millipore, Bedford, MA), and stained with Coomassie blue. The bands were cut out and subjected to the automatic N-terminal micro-sequencing at the Biochemistry Biotechnology Facility at Indiana University School of Medicine, Indianapolis, IN. In some experiments, the eluted PGN-binding proteins were separated by two-dimensional electrophoresis (isoelectric focusing and SDS–PAGE) [29], blotted onto Immobilon P, and sequenced as above.

2.4. Vancomycin-affinity chromatography and Western blots

Vancomycin coupled to agarose was used for sPGN affinity chromatography [28]. Vancomycin specifically binds to the D-Ala-D-Ala part of PGN, the structure uniquely found in non-cross-linked PGN and biosynthetic PGN precursors [28]. Tubulin (20 μg/100 μl, from Sigma) was incubated either in the polymerizing buffer (0.05 M phosphate or 0.1 M PIPES (pH 6.7) with 1 mM EGTA, 5 mM $MgCl_2$, and 1 mM GTP) with 20 μM taxol for 20 min at 37°C, or in the de-polymerizing buffer (0.05 M phosphate (pH 7.2) with 5 mM $CaCl_2$) for 1 h at 4°C, followed by 1 h incubation with 20 μg sPGN (labeled with [^{14}C]alanine) at 30°C for polymerizing conditions or at 20°C for de-polymerizing conditions. Tubulin polymerization is favored at 37°C in the presence of GTP and Mg^{2+} at $pH<7$ with no Ca^{2+}, and tubulin de-polymerization occurs at 4°C in the presence of Ca^{2+} at $pH>7$. The controls included sPGN alone or tubulin alone. Vancomycin–agarose columns (1 ml) were washed with either the polymerizing or de-polymerizing buffer with 0.1% Tween-20 at 20°C, the mixture of tubulin with sPGN in either polymerizing or de-polymerizing buffer was loaded on the column, the columns were washed with 12 ml of either the polymerizing or de-polymerizing buffer with 0.1% Tween-20, and the sPGN was eluted from the columns with NH_4OH in H_2O (pH 10) with 0.1% Tween-20, and collected into 0.6-ml fractions. The concentration of sPGN in the fractions was monitored by scintillation counting of ^{14}C and by absorbance at 218 nm. To detect the presence of tubulin, the fractions were concentrated by evaporation at 52°C, and subjected to SDS–PAGE on 11% gels, transferred to Immobilon P, and probed with a mixture of mouse anti-tubulin-α (clone B-5-1-2) and anti-tubulin-β (clone JDR.3B8) monoclonal antibodies (mAbs) (both reacting with tubulins from a variety of species, including mouse and bovine, obtained from Sigma), followed by incubation with peroxidase-labeled anti-mouse IgG, and detection by the enhanced chemiluminescence (ECL from Amersham, Chicago Heights, IL). The specificities and selectivities of the mAbs were confirmed using Western blots with purified tubulin and crude cell lysates (see Fig. 2B below).

In some experiments, cell lysates were used instead of tubulin. 15×10^6 RAW264.7 cells, grown in serum-free HL-1 medium, were washed twice with HBSS without Ca^{2+} and Mg^{2+}, and lysed by sonication in 200 μl of polymerizing buffer with 1.2% NP-40, 30 μg ml^{-1} aprotinin and 250 μg ml^{-1} PMSF on ice. Insoluble material was removed by centrifugation at $12\,000\times g$ at 4°C, and the supernatant was incubated with 1 mM GTP and 20 μM taxol at 37°C for 1.5 h, followed by incubation with sPGN and vancomycin affinity chromatography under polymerizing conditions, and by detection of sPGN and tubulin in the fractions, as described above. The controls included sPGN alone and cell lysate alone.

2.5. Binding of ^{125}I-labeled tubulin to sPGN–agarose and ReLPS–agarose

Tubulin (100 μg) and MAP-free tubulin (50 μg) were labeled with 2 mCi ^{125}I using pre-coated iodogen tubes (from Pierce, Rockford, IL) and checked for functional integrity [16]. Unbound ^{125}I was removed by dialysis at 4°C against 100 mM PIPES buffer (pH 6.9) with 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM GTP. The aliquots of the iodinated preparations were stored at −80°C and freshly thawed out for each experiment. The specific activities were 220–240 Ci $mmol^{-1}$ for tubulin, and 525–545 Ci $mmol^{-1}$ for MAP-free tubulin. Both ^{125}I-labeled tubulins yielded one 50 kDa band detected by autoradiography on

SDS–PAGE gels, that co-migrated with a Coomassie blue-stained band of unlabeled tubulin, confirming iodination of tubulin and not any potential contaminants, and minimal iodination of MAPs (see Fig. 3B below).

sPGN, ReLPS, smooth LPS, GlcNAc-MDP, MDP, or PGN-pentapeptide were coupled to 40–165 μm agarose beads [5], and the binding of iodinated tubulin to these agarose-immobilized preparations was performed as described before for the binding of ^{32}P-sCD14 [5]. For the standard binding assay under polymerizing conditions, [^{125}I]tubulin was pre-incubated in 10 μl of the polymerizing buffer with 1 mM GTP at 37°C for 20 min, and then diluted to 60 μl to yield final concentration of 1 μg ml^{-1} ([^{125}I]tubulin) or 0.5 μg ml^{-1} ([^{125}I]MAP-free tubulin), and incubated with 1.5 μl of sPGN–agarose (20 min with [^{125}I]tubulin, or 40 min with [^{125}I]MAP-free tubulin), or ReLPS–agarose (20 min for both [^{125}I]tubulins) at 37°C. Then PBS with 0.5 M NaCl was added, the agarose was centrifuged through 0.8 M sucrose, and the amount of ^{125}I bound to agarose was measured [5]. For the binding under de-polymerizing conditions, the assay was performed as above but in the de-polymerizing buffer at 4°C, with pre-incubation of [^{125}I]tubulin for 1 h, followed by incubation with sPGN–agarose or ReLPS–agarose for 3 h. In some experiments, the amount of agarose, incubation time, or tubulin concentrations were varied, as indicated in Section 3. For inhibition of binding, unlabeled tubulins were mixed with the iodinated tubulins and pre-incubated as described above, before addition of sPGN–agarose or ReLPS–agarose. In other competitive inhibition experiments, the competitors were first mixed with sPGN–agarose or ReLPS–agarose, followed by addition of iodinated tubulin. The apparent dissociation constant (K_d) and the maximal binding at saturation (B_{max}) were calculated as before [5].

2.6. Tubulin polymerization and electron microscopy

Tubulin (100 μl, 2 mg ml^{-1}) was incubated in the polymerizing buffer with 1 mM GTP, with or without 4 M glycerol, with or without 25 μg ml^{-1} of sPGN or ReLPS, and with or without 0.8 mM colchicine, at 37°C in 96-well microtiter plates, and the optical density (OD) at 405 nm was measured using a BioRad ELISA plate reader. Similar incubation in de-polymerizing buffer at 4°C did not cause an increase in OD (the OD values under de-polymerizing conditions were subtracted from the OD values under polymerizing conditions to obtain net OD). 25 μg ml^{-1} of sPGN and ReLPS was selected because in preliminary experiments it had the most consistent effect on microtubule polymerization (out of 2.5, 25, and 250 μg ml^{-1}). For electron microscopy, after 80 min of incubation in the polymerizing buffer with GTP, with or without glycerol, and with or without 25 μg ml^{-1} sPGN or ReLPS, samples were diluted 4 times, applied to carbon-coated nickel grids, stained with 2% uranyl acetate [17], and viewed in a JOEL-100CXII electron microscope.

3. Results

3.1. Tubulin is a major PGN-binding protein in cell lysates and membrane fractions of RAW264.7 macrophages

The major PGN-binding protein, consistently present in lysates of RAW264.7 cells and solubilized membrane fractions, that bound both to agarose-coupled sPGN or insoluble PGN, was β-tubulin (GenBank accession no. X04663) (Fig. 1). When similar samples were separated by two-dimensional electrophoresis (isoelectric focusing and SDS–PAGE), in addition to β-tubulin (which was the major spot), α-tubulin was also detected (not shown). CD14, another PGN-binding protein [5], could be detected by photoaffinity cross-linking [5] or on Western blots of PGN-binding proteins with anti-CD14 antibodies (not shown), but the amount of CD14 recovered from the cell lysates was not sufficient to yield a band that could be stained with Coomassie blue and sequenced.

3.2. Polymerized tubulin binds to sPGN and co-elutes with sPGN from anti-PGN vancomycin affinity column

When tubulin was incubated with sPGN under polymerizing conditions and then applied to a vancomycin–agarose column, both sPGN and tubulin were retained on the column and were then co-eluted at high pH (Fig. 2A,C, fractions 23 and 24). When tubulin was applied to the column by itself, it was not retained on the column. Sim-

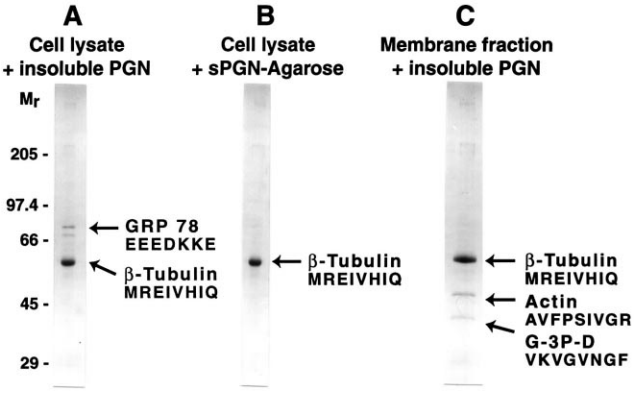

Fig. 1. Tubulin is a major PGN-binding protein in cell lysates and membrane fractions of RAW264.7 macrophages. Cell lysates (A,B) or membrane fraction (C) were incubated with insoluble PGN (A,C) or sPGN–agarose (B). PGN-binding proteins were resolved by SDS–PAGE, blotted onto Immobilon, and stained with Coomassie blue. The major band was identified by N-terminal sequencing as β-tubulin. Three other minor proteins, that bound to insoluble PGN, but not to sPGN, were GRP 78 (78 kDa glucose-regulated protein), actin, and glyceraldehyde-3-phosphate dehydrogenase (G-3P-D). The amino acid sequences are shown in the single-letter code, and the positions of molecular mass standards (kDa) are shown on the left.

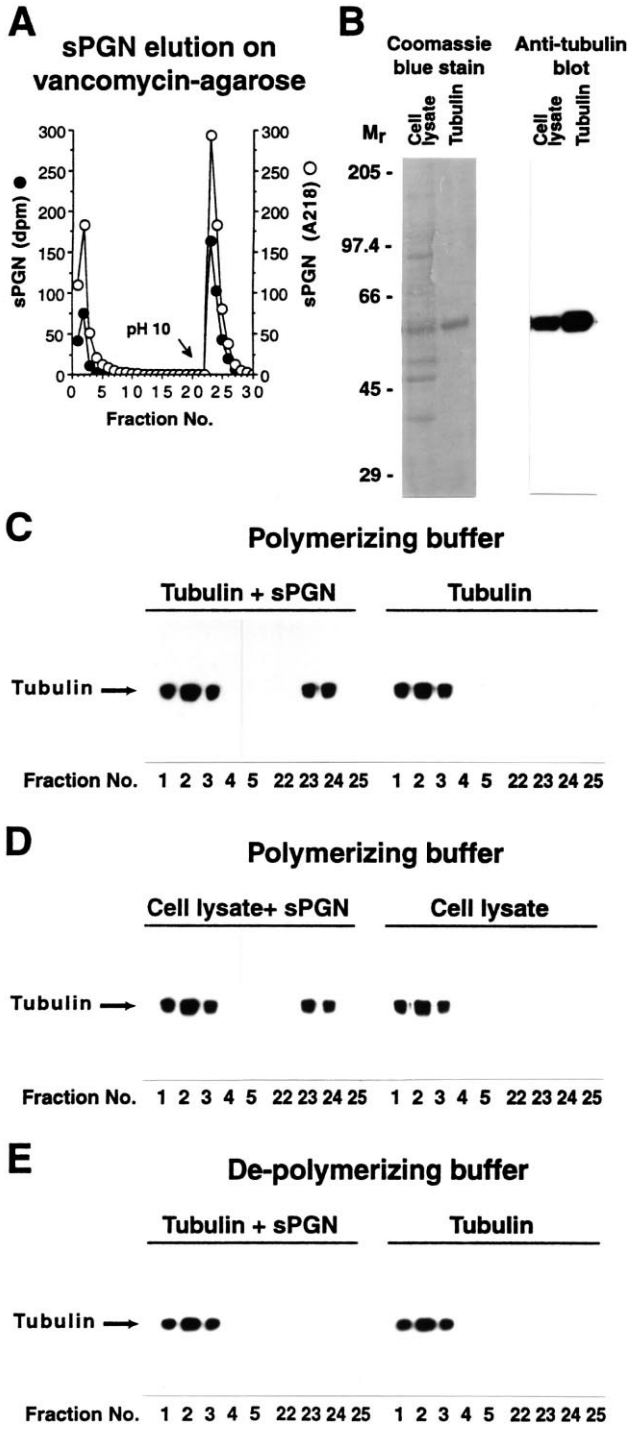

Fig. 2. Polymerized tubulin binds to sPGN and co-elutes with sPGN from anti-PGN vancomycin affinity column. Tubulin or RAW264.7 cell lysates were incubated with sPGN in the polymerizing or de-polymerizing buffer, applied onto vancomycin–agarose column, and washed. Vancomycin-bound sPGN (labeled with [^{14}C]alanine) eluted after application of pH 10 ammonia (fractions 23–25), as monitored by scintillation counting (dpm) and absorbance at 218 nm (A218) (A). Tubulin co-eluted with vancomycin-bound sPGN after application of pH 10 ammonia (fractions 23, 24), as demonstrated by Western blots of the fractions using anti-α- and β-tubulin mAbs, when either tubulin (C) or cell lysates (D) were incubated with sPGN in the polymerizing buffer, but did not co-elute with sPGN in the de-polymerizing buffer (E). Both sPGN and tubulin were also present in the void volume (fractions 1–3). Tubulin applied by itself to the vancomycin column eluted only in the void volume (C–E, fractions 1–3 on the right). All other fractions (not shown) did not contain tubulin. The specificity of anti-α- and β-tubulin mAbs was confirmed by comparing Western blots with the Coomassie blue staining of cell lysates and purified tubulin (B).
←

ilar results were obtained when lysates of RAW264.7 cells were used instead of purified tubulin (Fig. 2D). By contrast, under de-polymerizing conditions, tubulin was not retained with sPGN on the vancomycin–agarose column and was eluted only in the void volume, but not in the sPGN-containing fractions eluted with high pH (Fig. 2E). The amount of sPGN bound to vancomycin–agarose or its elution pattern were the same in all groups (not shown). These results indicate that tubulin binds to PGN preferentially under polymerizing conditions.

3.3. Comparison of the kinetics and affinity of binding of tubulin to PGN and LPS

Both tubulin and MAP-free tubulin bound to both sPGN–agarose and ReLPS–agarose, but not or very poorly to the agarose itself (Fig. 3A). The kinetics of bind-

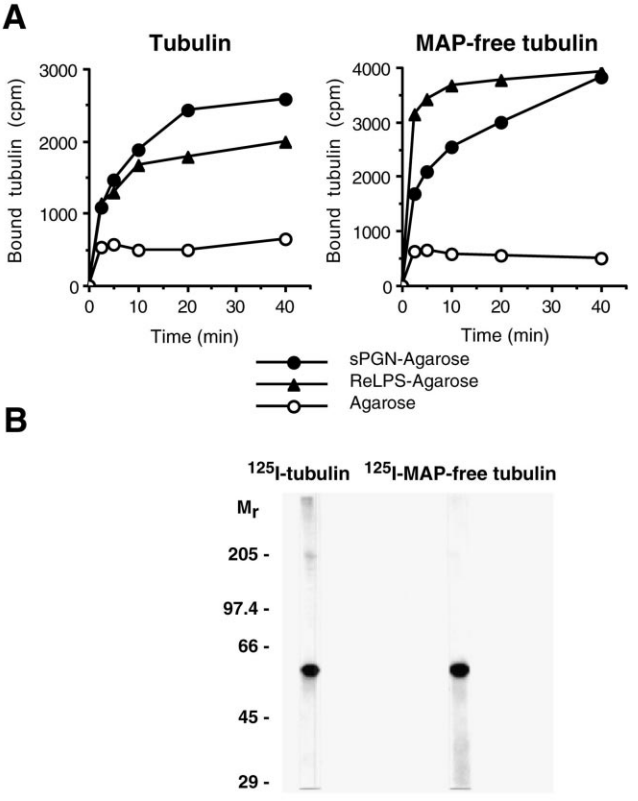

Fig. 3. Tubulin binds to sPGN–agarose and ReLPS–agarose: kinetics of binding. (A) [^{125}I]Tubulin (1 μg ml^{-1}) or [^{125}I]MAP-free tubulin (0.5 μg ml^{-1}) was incubated with 1 μl of sPGN–agarose, ReLPS–agarose, or control agarose in polymerizing buffer at 37°C, centrifuged through 0.8 M sucrose, and the amount of ^{125}I bound to agarose was measured. The results are means from three experiments; the S.E. were less than 15% and are not shown. (B) An autoradiogram of SDS–PAGE of [^{125}I]tubulin (0.1 μg/lane) or [^{125}I]MAP-free tubulin (0.05 μg/lane).

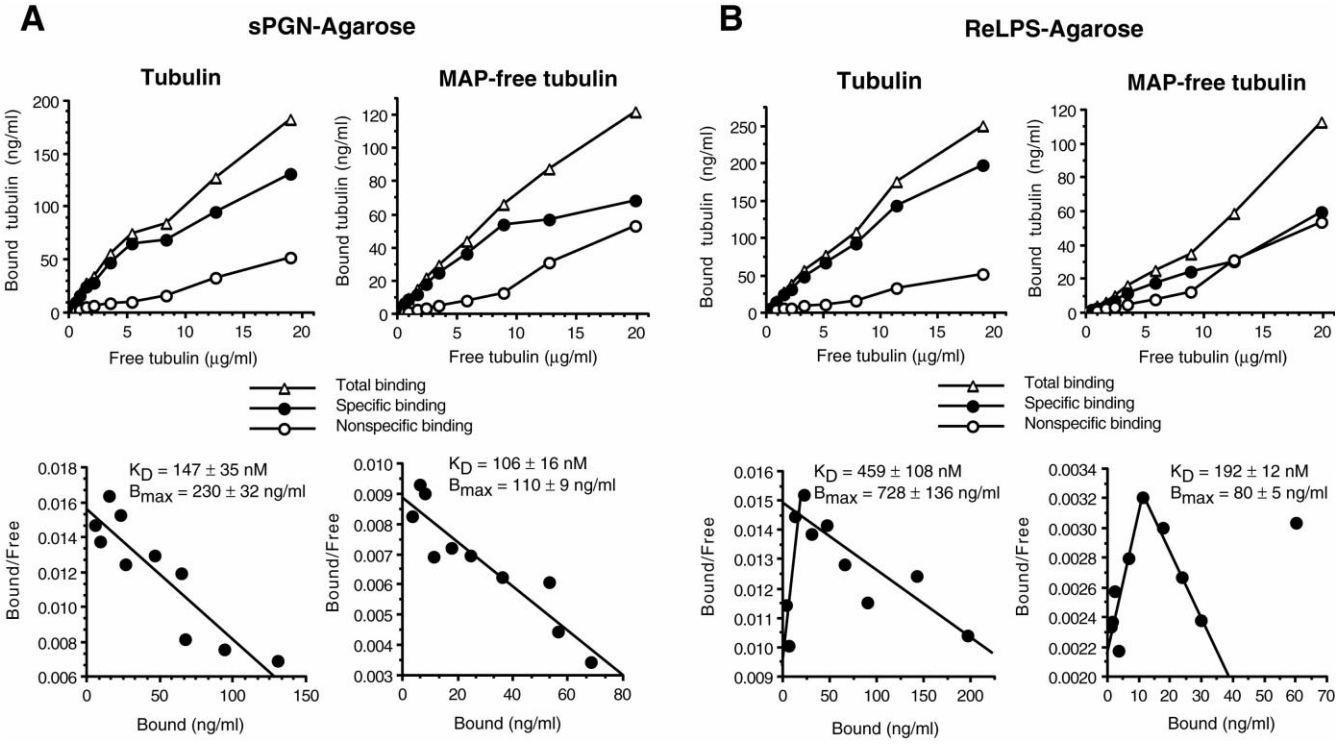

Fig. 4. Tubulin binds to sPGN–agarose with higher affinity than to ReLPS–agarose and binding of tubulin to ReLPS is cooperative. Increasing concentrations of [^{125}I]tubulin or [^{125}I]MAP-free tubulin were incubated with 1.5 μl of control agarose and sPGN–agarose (A) or ReLPS–agarose (B) in the polymerizing buffer, and the amounts of [^{125}I]tubulin bound to agarose and remaining unbound (free [^{125}I]tubulin) were measured. Total binding was the amount of ^{125}I associated with sPGN–agarose or ReLPS–agarose; non-specific binding was the amount of ^{125}I associated with control agarose; specific binding was total binding minus non-specific binding. The results are means of 4–5 experiments. The Scatchard plots were fitted using the Cricket Graph software ($r^2 = 0.82 \pm 0.024$, mean ± S.E.); mean apparent dissociation constants (K_d) and B_{max} (± S.E.) are also shown.

ing of tubulin to sPGN and ReLPS was similar, with most of the binding completed in 20 min. MAP-free tubulin bound to ReLPS with a much faster kinetics than to sPGN: binding to ReLPS was completed in 10 min, but to sPGN it required 40 min (Fig. 3A).

Both tubulin and MAP-free tubulin bound to sPGN in a saturable manner with similar affinity. The binding could be fitted to a hyperbolic curve and into a single straight line in a Scatchard plot, yielding an apparent $K_d = 147 \pm 35$ nM for tubulin and $K_d = 106 \pm 16$ nM for MAP-free tubulin (Fig. 4A). The B_{max}, however, was twice as high for tubulin than for MAP-free tubulin (230 ± 32 vs 110 ± 9 ng ml^{-1}).

In contrast to sPGN, binding of both tubulin and MAP-free tubulin to ReLPS showed cooperative binding (indicated by concave upwards Scatchard plots) (Fig. 4B). Also, the affinities of binding of tubulin and MAP-free tubulin to ReLPS (calculated from the descending slopes of the Scatchard plots) were lower than to sPGN. This difference in the affinity of binding to sPGN and ReLPS was a function of the ligand (tubulin) and not of a specific batch of sPGN–agarose or ReLPS–agarose, because similar results were obtained with two independently prepared batches of each agarose, and because the same agarose preparations previously [5] gave the expected higher affinity of binding of CD14 to ReLPS than to sPGN in the presence of LPS-binding protein.

Under de-polymerizing conditions, the amount of tubulin or MAP-free tubulin specifically bound to sPGN or ReLPS (B_{max}) decreased 10 or 5 times, respectively (data not shown). De-polymerizing conditions reduced the amount of microtubules by over 95% (determined by electron microscopy). These results explain why in Fig. 1 the binding was detected in the solubilizing buffer (which does not promote tubulin polymerization): binding under de-polymerizing conditions is 10 times lower than under polymerizing conditions, and in Fig. 1, cell lysate from 200 times more cells than in Fig. 2D was used.

3.4. Differential effects of unlabeled tubulin, sPGN, and ReLPS on the binding of tubulin to sPGN and ReLPS

Unlabeled tubulin or MAP-free tubulin competitively inhibited the binding of labeled [^{125}I]tubulin or [^{125}I]MAP-free tubulin to sPGN (Fig. 5). By contrast, the unlabeled tubulins not only did not inhibit the binding to ReLPS, but the addition of unlabeled tubulins or to [^{125}I]tubulins greatly enhanced the binding of labeled tubulins to ReLPS (Fig. 5). These results suggest that ReLPS induces or enhances tubulin polymerization (or

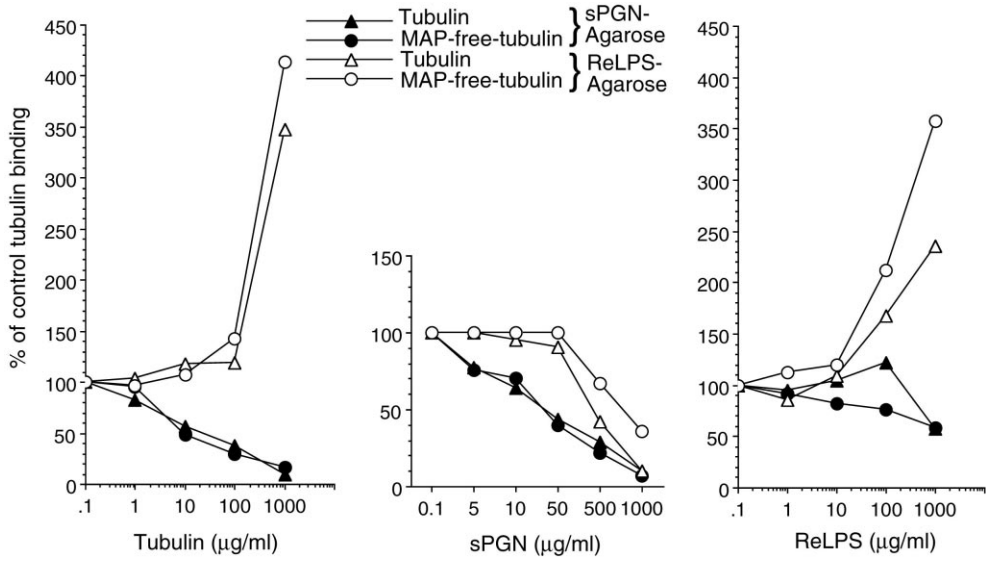

Fig. 5. Differential effects of unlabeled tubulin, sPGN, and ReLPS on binding of tubulin to sPGN–agarose and ReLPS–agarose. Binding of [^{125}I]tubulin or [^{125}I]MAP-free tubulin to sPGN–agarose or ReLPS–agarose in the polymerizing buffer in the presence of indicated concentrations of unlabeled tubulin or MAP-free tubulin, sPGN, or ReLPS was measured. The results were calculated as the percent of binding without a competitor and are means from three experiments; the S.E. were less than 18% and are not shown.

aggregation) that leads to continued increased binding of aggregated tubulin to ReLPS (or to tubulin bound to ReLPS). They also confirm the positive cooperative binding of tubulin to ReLPS (presented in Fig. 4B). sPGN (in solution) inhibited the binding of [^{125}I]tubulin to both sPGN and ReLPS (Fig. 5). ReLPS (in solution) increased the binding of [^{125}I]tubulin to ReLPS and showed only very weak inhibition of tubulin binding to sPGN at high concentrations (Fig. 5). These results further support the cooperative binding of tubulin to ReLPS.

3.5. *Macromolecular sPGN is required for binding to tubulin*

Neither tubulin nor MAP-free tubulin bound to any of the low molecular mass synthetic PGN fragments (MDP,

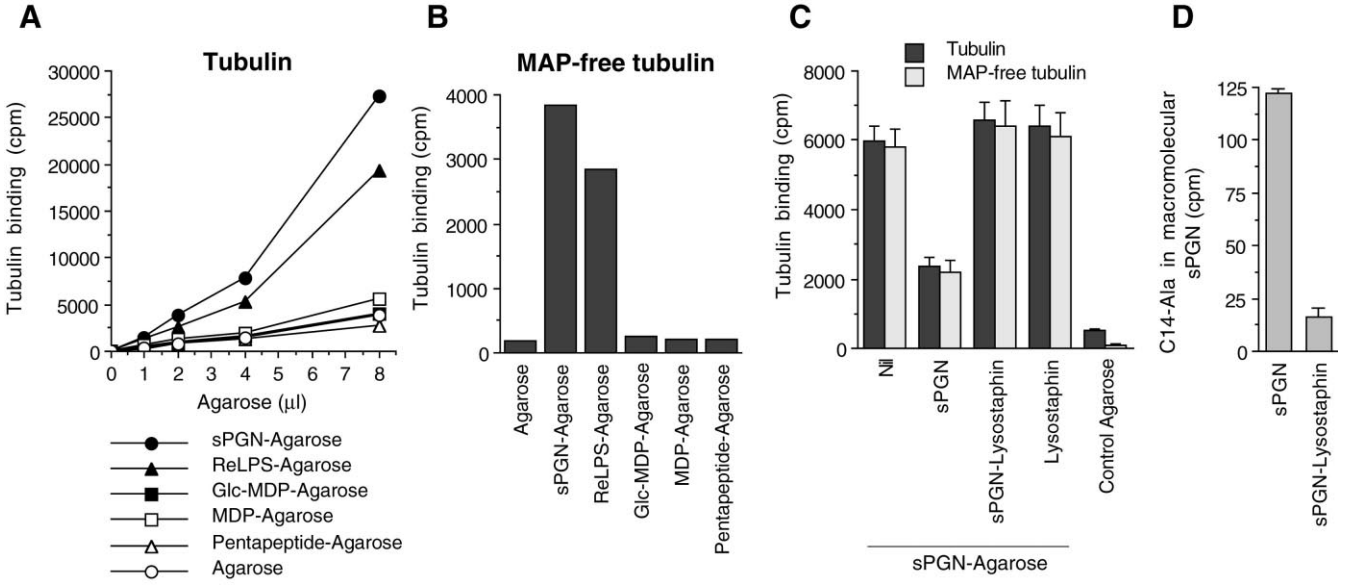

Fig. 6. Macromolecular sPGN is required for binding to tubulin. [^{125}I]tubulin (A) or [^{125}I]MAP-free tubulin (B) bound to sPGN–agarose, but not to low M_r sPGN fragments (GlcNAc-MDP, MDP, and PGN pentapeptide) coupled to agarose. The ability of sPGN (at 50 μg ml^{-1}) to inhibit the binding of tubulin to sPGN–agarose was lost after digestion of sPGN with lysostaphin and dialysis (C). The extent of digestion of sPGN was confirmed by the loss of [^{14}C]alanine from macromolecular biosynthetically labeled sPGN following lysostaphin digestion and dialysis (D). The results are from one of two similar experiments (A,B) or mean ± S.E. from four assays from two experiments (C,D).

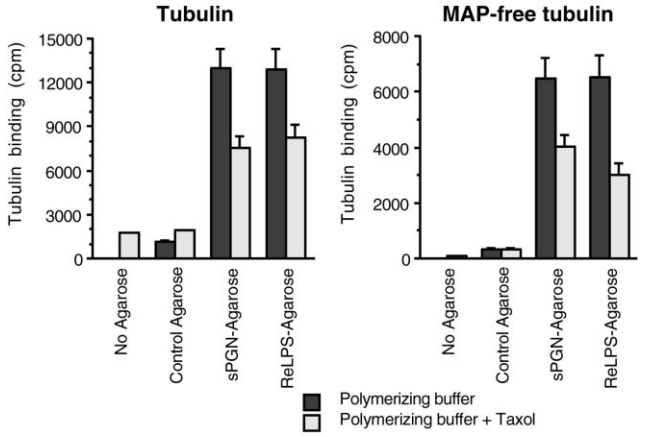

Fig. 7. Taxol reduces binding of tubulin to sPGN and ReLPS. [^{125}I]Tubulin or [^{125}I]MAP-free tubulin were pre-incubated without or with 20 μM taxol in the polymerizing buffer for 20 min at 37°C, before measuring the binding to 2 μl of control agarose, sPGN–agarose, or ReLPS–agarose (as described in Fig. 3). The results are means of four assays from two experiments ± S.E.

GlcNAc-MDP, and PGN pentapeptide) coupled to agarose (Fig. 6A,B). These synthetic low M_r PGN fragments, in contrast to high M_r polymeric PGN (Fig. 5), also did not inhibit tubulin binding to sPGN and ReLPS (data not shown). Morover, digestion of polymeric sPGN with a PGN-lytic enzyme, lysostaphin, abolished the ability of sPGN to competitively inhibit the binding of both tubulin and MAP-free tubulin to sPGN–agarose (Fig. 6C,D). These results suggest that polymeric PGN is required for the binding to tubulin.

3.6. Taxol diminishes binding of tubulin to sPGN and ReLPS

Taxol (an agent that stabilizes microtubules and promotes microtubule assembly) did not further increase the binding of tubulin and MAP-free tubulin to sPGN or ReLPS, but it diminished the binding of tubulin to both sPGN and ReLPS (Fig. 7).

3.7. Effect of sPGN and ReLPS on tubulin polymerization

In the presence of glycerol, sPGN (and to a smaller extent also ReLPS) significantly accelerated and enhanced tubulin polymerization (Fig. 8). However, in the absence of glycerol, both sPGN and ReLPS significantly diminished the overall extent of tubulin polymerization (Fig. 8). In all the groups, typical, uniform, straight, 30-nM-thick microtubules [17] were observed by electron microscopy (data not shown).

Tubulin polymerization in the absence or in the presence of either sPGN and ReLPS was similarly inhibited by 40 times molar excess of colchicine, an inhibitor of microtubule formation (by more than 80% without glycerol, or by more than 60% with 4 M glycerol, data not shown). These results indicate that the effect of sPGN and ReLPS was on true microtubule formation.

4. Discussion

We have discovered that PGN binds to tubulin. The binding is preferential under the conditions that promote tubulin polymerization, has an affinity of 100–150 nM, and does not require MAP. The affinity of binding of PGN to tubulin is 4–6 times lower than to CD14 [5].

LPS was previously found to bind to both purified tubulin (by photoaffinity cross-linking and co-elution on gel filtration) [23,24] and to microtubules (by electron microscopy) [24]. Our data confirm and further extend these results. We demonstrated that LPS binds both to tubulin and MAP-free tubulin with 2–3 times lower affinity than sPGN, but with a faster kinetics, and that in contrast to sPGN, binding of LPS shows positive cooperativity, indicating that LPS promotes tubulin aggregation.

PGN is composed of a glycan backbone of up to 100 alternating units of β-(1→4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), with short peptides linked to the lactyl group of the MurNAc residues. LPS is composed of lipid A, containing β-(1→6)-linked GlcNAc disaccharide, with phosphomonoester groups in positions 4′ and 1, amide- and ester-linked fatty acids linked to the remaining hydroxyl and amino groups, and core polysaccharide and *O*-polysaccharide chain linked to the C6′ hydroxyl group. The only similar parts of PGN and ReLPS molecules (that are, therefore, likely to participate in the binding to tubulin) are $(GlcNAc\text{-}MurNAc)_n$ backbone of PGN and $(GlcNAc)_2$ part of ReLPS, both containing closely located carbonyl residues.

Binding of PGN to tubulin requires polymeric PGN, and D-Ala-D-Ala of PGN pentapeptide is probably not directly involved in the binding. Moreover, ReLPS (natural minimum endotoxic structure, devoid of *O*-polysaccharide chain) binds to tubulin better than smooth LPS (data not shown). The PGN- and LPS-binding sites on tubulin

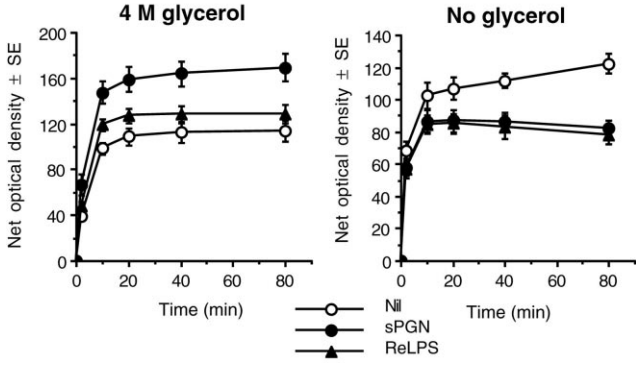

Fig. 8. Effect of sPGN and ReLPS on tubulin polymerization. Tubulin (2 mg ml^{-1}) was incubated in the polymerizing buffer with or without 4 M glycerol and with or without 25 μg ml^{-1} of sPGN or ReLPS, and OD_{405} was measured (means of four experiments).

are unknown, but they seem to be at least partially similar, because sPGN could competitively inhibit tubulin binding to both sPGN and ReLPS. However, the binding sites for sPGN and LPS are not identical, because sPGN was a better inhibitor for tubulin binding to sPGN than to ReLPS, although the affinity of binding of tubulin to sPGN was higher than to ReLPS. Similar binding sites for sPGN and ReLPS are also suggested by diminished binding of tubulin to both sPGN and ReLPS in the presence of taxol.

sPGN and ReLPS have a direct effect on tubulin polymerization in vitro. In the presence of glycerol, sPGN enhances tubulin polymerization significantly more than LPS whereas, without glycerol, both sPGN and LPS inhibit tubulin polymerization (Fig. 8). Our results without glycerol are, therefore, consistent with the previously observed fragmentation of microtubules induced by 10 times higher concentration of LPS in vitro [32]. Different effects in the medium with glycerol may be attributed to the stabilizing properties of glycerol for microtubules or its effect on MAP binding.

Although the involvement of microtubules in cell activation is not precisely known, microtubules serve as a scaffold for numerous signal transduction molecules that are associated with tubulin. For example, most of the LPS-activable mitogen-activated protein kinases in macrophages are associated with microtubules [14,15]. Moreover, several other key signal transduction molecules, such as G proteins [16,17,33], ZAP-70 kinase [34], Vav [34], Syk [35], phosphoinositide 3-kinase [36,37], phospholipase Cγ1 [36], c-Myc [38], and Rac1 [39] are associated with tubulin. Tubulin binds Src homology 2 domains (SH2) [36], and dynamin (one of MAPs) has binding sites for Src homology 3 domains (SH3) [40]. Both SH2 and SH3 are often present in signal transduction molecules. Furthermore, some cell surface receptors, e.g., glycine receptor [41] or CD2 [42], are associated with tubulin, and upon cell activation tubulin is phosphorylated on a single tyrosyl residue by Syk [35]. Therefore, these results collectively suggest that tubulin and microtubules are important participants in the cell activation processes. Because some of these proteins are involved in LPS- and PGN-induced cell activation [2,3,15,30,31,43], it is possible that tubulin may also play a role in LPS- and PGN-induced cell activation. This possibility needs to be investigated.

Because both PGN and LPS bind to tubulin and have a direct effect on tubulin polymerization, it is possible that PGN and LPS could interact with tubulin in the cell and modify microtubule function. Tubulin is located intracellularly mainly as a part of microtubules and also as a free protein pool. Therefore, extracellular ligands have to enter the cell to interact with tubulin. LPS is rapidly (within minutes) taken up by macrophages following interaction with membrane CD14, although LPS uptake may not be required for LPS-induced signal transduction [2,3]. However, recent results indicate that endocytic transport of LPS from the cell surface to the perinuclear Golgi apparatus is important for LPS-induced cell activation [25,26]. The exact mechanism of this transport is still unknown, but one of the well-established functions of microtubules is unidirectional transport of vesicles along microtubule tracks [13]. sPGN is also efficiently taken up by monocytes and macrophages (A.J. Ulmer, R. Dziarski, unpublished).

It is still not known how PGN or LPS would gain access to microtubules in the cells. It has been suggested that LPS can intercalate into the cytoplasmic or vesicle membranes [26] or that, similar to bacterial or plant toxins, LPS could cross the vesicle membranes into the cytoplasm [26]. Both mechanisms would give LPS an opportunity to interact with microtubules. It has been also suggested that tubulin may be exposed on the surface of monocytic cells and that LPS stimulation of these cells results in a marked reduction of the amount of cell surface-exposed tubulin [44]. This cell-surface tubulin would provide an opportunity for LPS and PGN to interact with tubulin before their internalization. However, the presence of cell-surface tubulin and its relevance to LPS- and PGN-induced cell activation need to be further confirmed. Now that the binding of PGN and LPS to tubulin and their effects on microtubules have been demonstrated, the mechanism of interaction of PGN and LPS with tubulin and microtubules in the cells needs to be investigated.

Acknowledgements

This work was supported by National Institute of Health Grants AI2879 (to R.D.) and MH39595 and AG15482 (to M.M.R.).

References

[1] B. Heymer, P.H. Seidl, K.H. Schleifer. Immunochemistry and biological activity of peptidoglycan, in: D.E.S. Stewart-Tull, M. Davis (Eds.), Immunology of the Bacterial Cell Envelope, Wiley, New York, 1985, pp. 11–46.

[2] R. Dziarski, A.J. Ulmer, D. Gupta, Interactions of bacterial lipopolysaccharide and peptidoglycan with mammalian CD14, in: R.J. Doyle (Ed.), Glycomicrobiology, Kluwer Academic/Plenum, New York, 2000, pp. 145–185.

[3] R. Dziarski, A.J. Ulmer, D. Gupta, Interactions of CD14 with components of Gram-positive bacteria, Chem. Immunol. 74 (2000) 83–107.

[4] D. Gupta, T.N. Kirkland, S. Viriyakosol, R. Dziarski, CD14 is a cell-activating receptor for bacterial peptidoglycan, J. Biol. Chem. 271 (1996) 23310–23316.

[5] R. Dziarski, R.I. Tapping, P.S. Tobias, Binding of bacterial peptidoglycan to CD14, J. Biol. Chem. 273 (1998) 8680–8690.

[6] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, C.J. Kirschning, Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2, J. Biol. Chem. 274 (1999) 17406–17409.

[7] A. Yoshimura, E. Lien, R.R. Ingalls, E. Tuomanen, R. Dziarski, D. Golenbock, Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2, J. Immunol. 163 (1999) 1–5.

[8] R.-B. Yang, M.R. Mark, A. Gray, A. Huang, M.H. Xie, M. Zhang, A. Goddard, W.I. Wood, A.L. Gurney, P.J. Godowski, Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling, Nature 395 (1998) 284–288.
[9] C.J. Kirschning, H. Wesche, T.M. Ayres, M. Rothe, Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide, J. Exp. Med. 188 (1998) 2091–2097.
[10] A. Poltorak, X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Riccardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene, Science 282 (1998) 2085–2088.
[11] K. Hoshino, O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, S. Akira, Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product, J. Immunol. 162 (1999) 3749–3752.
[12] S.D. Wright, Toll, a new piece in the puzzle of innate immunity, J. Exp. Med. 189 (1999) 605–609.
[13] L. Stryer, Biochemistry, 4th ed., W.H. Freeman, New York, 1995, pp. 405–411.
[14] A.A. Reszka, R. Seger, C.D. Diltz, E.G. Krebs, E.H. Fischer, Association of mitogen-activated protein kinase with the microtubule cytoskeleton, Proc. Natl. Acad. Sci. USA 92 (1995) 8881–8885.
[15] A. Ding, B. Chen, M. Fuortes, E. Bloom, Association of mitogen-activated protein kinases with microtubules in mouse macrophages, J. Exp. Med. 183 (1996) 1899–1904.
[16] N. Wang, K. Yan, M.M. Rasenick, Tubulin binds specifically to the signal-transducing proteins, $G_{s\alpha}$ and $G_{i\alpha}1$, J. Biol. Chem. 265 (1990) 1239–1242.
[17] S. Roychowdhury, M.M. Rasenick, G protein $\beta1\gamma2$ subunits promote microtubule assembly, J. Biol. Chem. 272 (1997) 31576–31581.
[18] A.H. Ding, F. Porteu, E. Sanchez, C.F. Nathan, Shared actions of endotoxin and taxol on TNF receptors and TNF release, Science 248 (1990) 370–372.
[19] A. Ding, E. Sanchez, C.F. Nathan, Taxol shares the ability of bacterial lipopolysaccharide to induce tyrosine phosphorylation of microtubule-associated protein kinase, J. Immunol. 151 (1993) 5596–5602.
[20] C.L. Manthey, M.E. Brandes, P.Y. Perera, S.N. Vogel, Taxol increases steady-state levels of lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation in murine macrophages, J. Immunol. 149 (1992) 2459–2465.
[21] C.L. Manthey, N. Qureshi, P.L. Stutz, S.N. Vogel, Lipopolysaccharide antagonists block taxol-induced signaling in murine macrophages, J. Exp. Med. 178 (1993) 695–702.
[22] P.-Y. Perera, N. Qureshi, S.N. Vogel, Paclitaxel (taxol)-induced NF-κB translocation in murine macrophages, Infect. Immun. 64 (1996) 878–884.
[23] A. Ding, E. Sanchez, M. Tancino, C. Nathan, Interactions of bacterial lipopolysaccharide with microtubule proteins, J. Immunol. 148 (1992) 2853–2858.
[24] C. Risco, J.E. Dominguez, M.A. Bosch, J.L. Carrascosa, Biochemical and electron microscopy analysis of the endotoxin binding to microtubules in vitro, Mol. Cell. Biochem. 121 (1993) 67–74.
[25] N. Thieblemont, R. Thieringer, S.D. Wright, Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement, Immunity 8 (1998) 771–777.
[26] N. Thieblemont, S.D. Wright, Transport of bacterial lipopolysaccharide to the Golgi apparatus, J. Exp. Med. 190 (1999) 523–534.
[27] I. Ringel, S.B. Horwitz, Effect of alkaline pH on taxol-microtubule interactions, J. Pharmacol. Exp. Ther. 259 (1991) 855–860.
[28] R.S. Rosenthal, R. Dziarski, Isolation of peptidoglycan and soluble peptidoglycan fragments, Methods Enzymol. 235 (1994) 253–286.
[29] R. Dziarski, Demonstration of peptidoglycan-binding sites on lymphocytes and macrophages by photoaffinity cross-linking, J. Biol. Chem. 266 (1991) 4713–4718.
[30] D. Gupta, Y. Jin, R. Dziarski, Peptidoglycan induces transcription and secretion of TNF-α and activation of Lyn, extracellular signal-regulated kinase, and Rsk signal transduction proteins in mouse macrophages, J. Immunol. 155 (1995) 2620–2630.
[31] R. Dziarski, Correlation between ribosylation of pertussis toxin substrates and inhibition of peptidoglycan-, muramyl didpeptide- and lipopolysaccharide-induced mitogenic stimulation in B lymphocytes, Eur. J. Immunol. 19 (1989) 125–130.
[32] K.J. Bohm, W. Vater, S. Russwurm, K. Reinhart, E. Unger, Lipopolysaccharide-caused fragmentation of individual microtubules in vitro observed by video-enhanced differential interference contrast microscopy, FEBS Lett. 425 (1998) 134–136.
[33] S. Roychowdhury, N. Wang, M.M. Rasenick, G protein binding and G protein activation by nucleotide transfer involve distinct domains on tubulin: regulation of signal transduction by cytoskeletal elements, Biochemistry 32 (1993) 4955–4961.
[34] R.D.J. Huby, G.W. Carlile, S.C. Ley, Interactions between the protein-tyrosine kinase ZAP-70, the proto-oncoprotein Vav, and tubulin in Jurkat T cells, J. Biol. Chem. 270 (1995) 30241.
[35] J.D. Peters, M.T. Furlong, D.J. Asai, M.L. Harrison, R.L. Geahlen, Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates α-tubulin on tyrosine, J. Biol. Chem. 271 (1996) 4755–4762.
[36] T. Itoh, K. Miura, H. Miki, T. Takenawa, β-tubulin binds Src homology 2 domains through a region different from the tyrosine-phosphorylated protein-recognizing site, J. Biol. Chem. 271 (1996) 27931–27935.
[37] R. Kapeller, A. Toker, L.C. Cantley, C.L. Carpenter, Phosphoinositide 3-kinase binds constitutively to α/β-tubulin and binds to γ-tubulin in response to insulin, J. Biol. Chem. 270 (1995) 25985–25991.
[38] N. Alexandrova, J. Niklinski, V. Bliskovsky, G.A. Otterson, M. Blake, F.J. Kaye, M. Zajac-Kaye, The N-terminal domain of c-Myc associates with α-tubulin and microtubules in vivo and in vitro, Mol. Cell. Biol. 15 (1995) 5188–5195.
[39] A. Best, S. Ahmed, R. Kozma, L. Lim, The Ras-related GTPase Rac1 binds tubulin, J. Biol. Chem. 271 (1996) 3756–3762.
[40] J.S. Herskovits, H.S. Shpetner, C.C. Burgess, R.B. Vallee, Microtubules and Src homology 3 domains stimulate the dynamin GTPase via its C-terminal domain, Proc. Natl. Acad. Sci. USA 90 (1993) 11468–11472.
[41] J. Kirsch, D. Langosch, P. Prior, U.Z. Littauer, B. Schmitt, H. Betz, The 93-kDa glycine receptor-associated protein binds to tubulin, J. Biol. Chem. 266 (1991) 22242–22245.
[42] R. Offringa, B.E. Bierer, Association of CD2 with tubulin: Evidence for a role of the cytoskeleton in T cell activation, J. Biol. Chem. 268 (1993) 4979–4988.
[43] Y. Jin, D. Gupta, R. Dziarski, Endothelial and epithelial cells do not respond to complexes of peptidoglycan with soluble CD14, but are activated indirectly by peptidoglycan-induced tumor necrosis factor-α and interleukin-1 from monocytes, J. Infect. Dis. 177 (1998) 1629–1638.
[44] S.B. Por, M.A. Cooley, S.N. Breit, R. Penny, P.W. French, Antibodies to tubulin and actin bind to the surface of a human monocytic cell line, U937, J. Histochem. Cytochem. 39 (1991) 981–985.