Chlamydia species are obligate intracellular pathogens that utilize a type three secretion system to manipulate host cell processes. Genetic manipulations are currently not possible in Chlamydia species, necessitating study of effector proteins in heterologous expression systems and severely complicating efforts to relate molecular strategies used by Chlamydia to the biochemical activities of effector proteins. CopN is a chlamydial type three secretion effector that is essential for virulence. Heterologous expression of CopN in cells results in loss of microtubule spindles and metaphase plate formation and causes mitotic arrest. CopN is a multidomain protein with similarity to type three secretion effector proteins. CopN binds directly to actin but not to microtubules (MTs). Furthermore, CopN inhibits tubulin polymerization by sequestering free $\alpha$-tubulin, similar to one of the mechanisms utilized by stathmin. Although CopN and stathmin share no detectable sequence identity, both influence MT formation by sequestration of $\alpha$-tubulin. CopN displaces stathmin from preformed stathmin-tubulin complexes, indicating that the proteins bind overlapping sites on tubulin. CopN is the first bacterial effector shown to disrupt MT formation directly. This recognition affords a mechanistic understanding of a strategy Chlamydia species use to manipulate the host cell cycle.

Chlamydia species, the causative agents of a range of human diseases, including genital, ocular, and respiratory infection (3, 14–16), are obligate intracellular pathogens with a biphasic life cycle consisting of metabolically inactive elementary bodies and metabolically active reticulate bodies (17). Infection involves attachment and cellular uptake of elementary bodies and subsequent differentiation into reticulate bodies. The reticulate bodies remain in endosome-derived membrane vacuoles termed “inclusions,” which migrate to the microtubule-organizing center in a dynein-dependent process (18–20). After multiple rounds of bacterial cell division, the reticulate bodies re-differentiate into infectious elementary bodies and spread to neighboring cells (17). Chlamydia species are intractable to genetic manipulation, complicating the task of assigning function to effector proteins and, hence, the development of a mechanistic understanding of interactions between Chlamydia and the host (3). As with other Gram-negative bacteria, Chlamydia species use a type three secretion system (T3SS) and T3SS effectors to subvert host defenses and reprogram cell processes (1–3).

Type three secretion is a complex pathway used by many Gram-negative bacteria to deliver effector proteins to their hosts (for extensive reviews, see Refs. 21 and 22). The T3SS is composed of a needle complex (or type three secretion apparatus), an ATPase, translocator molecules, effector molecules, and chaperones. The needle complex is a large multiprotein complex that spans bacterial membranes and serves as a conduit through which secreted proteins reach the target cell. Proteins are secreted through this pore in an ATP-consuming process using specialized ATPases located in the bacterial cytosol, at the base of the needle complex. Secreted proteins include translocators, which are membrane proteins that form a pore in the host membrane, and effectors, which are a diverse group of proteins that subvert host cell processes and orchestrate bacterial adhesion or engulfment (among other activities). Within the bacterial cytosol, effectors and translocators are often found in complex with specialized chaperones that keep them in a secretion-compotent state until secretion is initiated. External cues initiate secretion, at which time a plug protein that had been occluding the pore is secreted, and the T3SS becomes active. A common target for T3SS effectors is the host’s cytoskeleton, which is frequently remodeled during infection (3, 21, 23–26).

Bacterial pathogens typically secrete effectors that modulate the actin cytoskeleton, which enables remodeling of the membrane to promote uptake or adhesion of the bacteria. The tubulin cytoskeleton is less frequently the target of effectors, although there are some reports describing effectors that target tubulin or microtubules (MTs) (4, 27). MTs are critical eukaryotic cytoskeletal structures (reviewed in Ref. 28) composed principally of $\alpha$-tubulin and decorated with MT accessory proteins.

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*This work was supported, in whole or in part, by National Institutes of Health Grant UL1RR024975-01 from the National Center for Research Resources and Grant DK058404 (to B. W. S.) and Grant NIH 5T90 DA022873 (to T. L. A.). The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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2 The abbreviations used are: T3SS, type three secretion system; MT, microtubule; GMP-CPP, guanosine 5’-($\alpha$,\beta-methylene)triphosphate.
proteins. In non-dividing cells, they form a structural network used, in combination with MT-associated motor proteins, for vesicle trafficking and organelle localization. In motile cells, they are essential components of cilia and flagella. During cell division, MTs form the mitotic spindle and segregate the chromosomes to daughter cells.

A prominent feature of infection is that Chlamydia species modulate the host cell cycle, resulting in delayed cytokinesis and failure to segregate sister chromosomes properly (4, 18, 29). Although the advantage afforded to the pathogen is not clear, two mechanistic strategies for affecting the cell cycle have been identified: regulation of the G2/M transition by cleavage of mitotic cyclin B1 (29) and a CopN-dependent mitotic arrest (4). Expression of CopN in eukaryotic cells results in severe alterations in the MT network, including destruction of mitotic spindles (4). CopN is a putative T3SS plg proteoglycan (1, 30). Plg proteins typically block the secretion pore, allowing pathogen export only in the presence of an unknown extracellular signal (31–33). The discoveries that CopN is a T3SS effector (1) that destroys MT networks (1, 4) as well as a putative plug protein (30, 34) motivated us to determine the biochemical activity of CopN. We show that CopN binds directly to αβ-tubulin and inhibits tubulin polymerization into MTs.

MATERIALS AND METHODS

Purification of His-tagged Proteins—Gateway expression vectors for CopN from Chlamydia pneumoniae, Chlamydia trachomatis, and Chlamydia psittaci B577 (Chlamydia pneumoniae abortus) were described previously (4). C. pneumoniae CopN was subsequently cloned by standard PCR methods into pET28, thus incorporating an N-terminal hexahistidine tag, and all truncations were made in pET28 using PCR-based mutagenesis. Scc2 and Scc3 were PCR-amplified from genomic DNA (ATCC strain AR-39) and cloned into pET28 with an N-terminal hexahistidine tag. Human stathmin was subcloned from a pET15 vector into pET28 and fused to an N-terminal hexahistidine tag. Proteins were expressed in BL21(DE3) cells grown in LB medium at 37 °C and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 12 h at 20 °C. Bacteria were harvested by centrifugation and lysed with a French press, and proteins were purified by metal affinity chromatography and, for all proteins except stathmin, gel filtration. Proteins were snap-frozen in liquid nitrogen and stored at −80 °C until needed.

Tubulin Purification—Fresh bovine brains were obtained from a local slaughterhouse (C & F Meat Co., College Grove, TN), and tubulin was prepared as described (35), with the only modifications being that 600 g of bovine brains rather than 1 kg of porcine brains were used (buffer volumes were adjusted accordingly) and that the rotors used were JA-10, Ti-45, and Ti-70.1 rather than SLA 1500, Ti-45, and TLA 100.4 (rotor velocities were adjusted to achieve appropriate g forces). Tubulin was snap-frozen in BRB80 buffer (80 mM PIPES, 1 mM MgCl2, and 1 mM EGTA, pH 6.8) in liquid nitrogen and stored at −80 °C until needed.

Gel Filtration Assays—Tubulin and chaperone binding assays were performed by gel filtration using a 24-ml Superdex S200 column (GE Healthcare), equilibrated in S200 buffer (10 mM Tris-HCl and 150 mM NaCl, pH 7.5), run at 0.5 ml/min, and maintained at 4 °C. CopN and tubulin or CopN and Scc3 were mixed, allowed to form complexes for 15 min, and applied to the column. For the stathmin–tubulin–CopN experiments, stathmin and tubulin were mixed first and allowed to equilibrate for 15 min, and then CopN was added and allowed to equilibrate for an additional 15 min.

MT Pelleting Assays—MT pelleting assays were performed essentially as described (12, 36). Taxol-stabilized MTs (10 μg/reaction) were incubated with CopNΔ8 (2, 6, 10, 20, 30, and 40 μg/reaction) for 15 min at 25 °C and spun through a 60% sucrose cushion (20 min at 140,000 × g in a TLA 100.4 rotor). Fractions from the supernatant and pellet were analyzed by SDS-PAGE. Human stathmin (5 μg/reaction) and the rat MAP2c (microtubule-associated protein 2c) MT-binding domain (20 μg/reaction; a gift from Kevin Slep, University of North Carolina at Chapel Hill) (37) were used as positive and negative controls for MT binding, respectively.

Turbidity Assays—Turbidity assays were performed in triplicate in a BioTek Synergy 4 plate reader in 96-well plates. Each assay well included 200 μg of tubulin, 3 mM GTP, 80 mM PIPES, 2 mM MgCl2, 0.5 mM EGTA, and 30% glycerol in a total volume of 300 μl. Plates were set up on ice, mixed, and transferred to an incubated plate reader maintained at 37 °C. Absorbance at 340 nm was measured at 45-s intervals, and the plates were shaken for 5 s before each measurement. The data presented are the average of three replicate wells.

MT Disassembly—MT disassembly was assayed essentially as described (12, 36, 38). Surfaces of double-stick tape flow cells were coated with 1 mg/ml biotinylated BSA followed by 0.1 mg/ml streptavidin. 1 mM fluorescent (1:9 rhodamine-labeled, unmodified) and biotinylated (1:100 biotinylated:unmodified) GMP-CPP-stabilized MTs were then bound to the flow cell surface and washed with flow cell buffer (80 mM PIPES, pH 6.8, 0.5 mM MgCl2, and 1 mM EGTA containing 1 mM MgATP, 500 mg/ml casein, 50 mM KCl, and an oxygen scavenging mixture) (39). CopN was diluted to 2.8 μM in flow cell buffer and added to the flow cell. MT disassembly was monitored by time-lapse fluorescence microscopy using a Nikon 90i epifluorescence microscope with a 100× 1.4 numerical aperture Nikon objective and a CoolSNAP HQ2 cooled CCD camera. MT lengths were measured at 0 and 4 min. These experiments were performed simultaneously with experiments presented by Du et al. (36); therefore, the control experiments represent the same set of controls reported in that publication.

Electron Microscopy—GMP-CPP-stabilized MTs were prepared as described (40). MTs were mixed with buffer alone (BRB80), the MAP2c MT-binding domain (a known MT-binding protein (37)), or CopN and incubated for 5 min at room temperature before being prepared for imaging by conventional negative stain electron microscopy. Uranyl formate-stained samples were prepared for electron microscopy as described (41). In brief, the sample (3 μl) was absorbed onto a glow-discharged 200-mesh copper grid covered with carbon-coated collodion film. The grid was washed with two drops of water and then stained with two drops of uranyl formate (0.75%). Samples were imaged on an FEI Morgagni electron microscope operated at an acceleration voltage of 100 kV.
CopN Sequesters Tubulin and Prevents Microtubule Assembly

Images were recorded at a magnification of either ×11,000 or ×36,000 and collected using a 1000 × 1000 CCD camera (ATM).

Competitive Binding Assay—This assay was performed analogously to a competitive ELISA. Wells in a 96-well microtiter plate were coated with 1 µg/well tubulin in BRB80 buffer for 10 h at 4 °C. The plate was subsequently washed two times with PBS, blocked for 2 h with PBS and 3% BSA at 22 °C, and washed four times with PBS. Biotinylated stathmin, labeled with sulfo-NHS-biotin (Thermo Scientific) following the manufacturer’s protocol and using a 40-fold molar excess of sulfo-NHS-biotin, was added simultaneously with unlabeled CopN to each well. Biotinylated stathmin (100 µl of 2.0 µM) was added to each well. This value was the EC_{50} of biotinylated stathmin in experiments without CopN. CopN (100 µl) was added as a dilution series from 10 to 0.020 µM. To add these reagents simultaneously and to avoid order of addition kinetics, they were pre-mixed in a 96-well plate with very low protein binding in PBS, 3% BSA, and 0.05% Tween 20. Binding was for 2 h at 22 °C, followed by four PBS washes. The biotin signal was developed with alkaline phosphatase-linked streptavidin using reagents from Kirkegaard & Perry Laboratories and read on a BioTek Synergy 4 plate reader at 405 nm. Control experiments included conditions without tubulin, without CopN, and without biotinylated stathmin.

RESULTS

Domain Mapping Experiments Demonstrate That CopN Encodes Three Domains—CopN, which is found in all Chlamydia species, shows weak sequence homology to a family of bacterial proteins that function as plug proteins for the T3SS (30). This family includes YopN from Yersinia pestis and MxiC from Shigella. Plug proteins exist in two different organizational structures, a YopN-like form in which the plug and a regulator protein (TyeA in the case of YopN) exist as two separate polypeptides (31) and a single polypeptide form exemplified by MxiC, the T3SS plug protein from Shigella (32). In MxiC, a TyeA-like domain is present in the C-terminal region of the protein (33). CopN is ∼100 amino acids longer than YopN (399 versus 293 amino acids) and ∼50 amino acids longer than MxiC (399 versus 354 amino acids) and has been proposed to be a MxiC-like plug protein (30).

As a first step in our biochemical characterization of CopN, we mapped its domain structure by limited proteolysis and mass spectrometry using trypsin and chymotrypsin. This approach, in which unstructured regions are rapidly degraded, identifies domains based on protease resistance. Our results, summarized in Fig. 1, indicate that CopN is composed of a flexible N terminus, a protease-resistant central core, and a protease-resistant C-terminal domain. Despite very low sequence identity (45 conserved residues between CopN and MxiC), this domain structure is similar to the domain organization of MxiC (33). This domain structure has two implications: first, that amino acids 85–268 form a plug domain that is structurally similar to YopN and the plug region of MxiC, and second, that the C-terminal ∼130 amino acids of CopN form a regulatory domain, perhaps similar to TyeA and the C-terminal domain of MxiC, which controlsCopN secretion and hence activation of the T3SS. In the case of Y. pestis, TyeA binds the C terminus of YopN and prevents YopN secretion until an unknown signal promotes the release of TyeA (31). In the case of CopN and other MxiC-like plug proteins, secretion is likely triggered by a conformational change within the C-terminal domain rather than by release of a regulatory protein.

CopN Binds Scc3, a Class II Chaperone, Using Regions outside of the Unstructured N Terminus—The N termini of type three secretion effectors are unstructured and function to promote secretion, with some binding cognate chaperones and becoming partially ordered upon binding (22, 42–44). To determine whether the N-terminal region of CopN binds a chaperone, we first identified the relevant chaperone. The candidate chaperone, Scc3, which was shown to bind CopN in yeast and bacterial two-hybrid experiments (34, 45), was cloned, expressed, and purified. Using purified components, we found that Scc3 promoted a shift of CopN, as well as an N-terminal truncation of CopN (CopNΔ84), to a faster elution time on a gel filtration column and that, when added together, the proteins co-eluted at the faster time, indicating that Scc3 binds directly to CopN (Fig. 1 and supplemental Fig. 1).

CopN Binds Directly to Tubulin but Not to MTs—Heterologous expression of C. pneumonia CopN in eukaryotic cells results in disruption of mitotic spindles, which are composed of MTs, and mitotic arrest (4). Such characteristics are consistent with the action of a MT-destabilizing protein, motivating us to investigate the possibility that CopN binds tubulin or MTs. Using purified components, we determined that CopN bound directly to αβ-tubulin. Using gel filtration to measure protein-protein interactions as a shift in retention time, we found that CopN significantly retarded the retention time for αβ-tubulin (Fig. 2A and supplemental Fig. 2, A and B), consistent with a
CopN Sequesters Tubulin and Prevents Microtubule Assembly

FIGURE 2. CopN binds tubulin but not MTs. A–C, gel filtration-based binding assays in which proteins were run individually and as a mixture on an analytical gel filtration column. In these traces, CopN is red, tubulin is blue, and the complex is green. A and B, CopN + tubulin ran faster than either individual component, indicating that the purified proteins directly associate. C, C. trachomatis (C. tr.) CopN did not bind tubulin tightly enough to survive gel filtration. C. pn., C. pneumoniae; C. ps., C. psittaci; mA.U., milli-absorbance units. D, MT pelleting assay. In this assay, Taxol-stabilized MT were prepared; mixed with C. pneumoniae CopN, stathmin, or MAP2c; and fractionated between a soluble (S) tubulin-containing fraction and an insoluble (P) MT-containing fraction by centrifugation. CopN failed to co-pellet with MTs, indicating that it does not bind MTs. Taken together with A and B, these data indicate direct binding between CopN and tubulin but not between CopN and MTs.

model in which the disruption of mitotic spindles observed when CopN is expressed is mediated by direct tubulin binding. We next investigated whether additional homologs bound αβ-tubulin. As shown in Fig. 2B and supplemental Fig. 2C, CopN from C. psittaci B577 (C. abortus), but not from C. trachomatis (Fig. 2C and supplemental Fig. 2D), interacted with αβ-tubulin by gel filtration.

To determine whether CopN binds to both MTs and tubulin or if it is specific for unpolymerized tubulin, we evaluated the ability of CopN to bind MTs using a MT pelleting assay. In this assay, CopN was incubated with MTs, which were subsequently separated from free αβ-tubulin by centrifugation. This assay is typically used to show binding to MTs, in which case, the binding partner is pulled to the pellet with the MTs. As shown in Fig. 2D, CopN remained in the supernatant, indicating that it does not bind MTs. Although this experiment cannot rule out that a small amount of CopN binds to MTs, taken in its entirety, Fig. 2 shows that CopN has a clear preference for unpolymerized tubulin, indicating that it binds a surface of tubulin that is inaccessible or altered in MTs. We further evaluated the possibility that CopN might induce bundling or some other alteration of MTs. Negative stain electron microscopy demonstrated that CopN did not alter MT structure (supplemental Fig. 3).

CopN Inhibits MT Polymerization but Does Not Depolymerize MTs—The direct interaction between CopN and αβ-tubulin and the lack of a detectable interaction between CopN and MTs led us to hypothesize that CopN promotes MT instability by binding and sequestering αβ-tubulin, thus preventing polymerization, i.e. CopN might sequester αβ-tubulin and effectively raise the critical concentration of assembly for tubulin. Stathmins, a eukaryotic tubulin-binding protein, is known to use an analogous “sequestration” mechanism to prevent tubulin polymerization (7–11). To evaluate how tubulin binding by CopN alters MT polymerization, we optimized a standard MT turbidity assay (12) for a 96-well plate. In this assay, tubulin is incubated at 37 °C and allowed to polymerize into MTs, which scatter light. Absorbance at 350 nm is measured as an indicator of polymerization. Use of a 96-well format allowed us to compare all proteins directly in very uniform conditions. CopN from C. pneumoniae and C. psittaci strongly inhibited tubulin polymerization, whereas CopN from C. trachomatis showed weaker but measurable inhibition (Fig. 3A). CopN from C. pneumoniae was particularly effective at inhibiting MT formation, showing near-complete inhibition at 25 μM. Consistent with the observation that CopN binds free αβ-tubulin but not MTs, we also observed that it inhibited αβ-tubulin polymerization but did not promote depolymerization of MTs. We evaluated the ability of CopN to destabilize MTs by mixing CopN with preformed rhodamine-labeled MT as described (36, 38). As shown in Fig. 3B, no change in MT size was evident following incubation with CopN.

The similar capacities of both CopN and stathmin to bind free tubulin and inhibit polymerization motivated us to determine whether CopN is a distant prokaryotic stathmin homolog. Stathmins contain multiple copies of an ~35-amino acid tubulin-binding repeat, each of which binds a single αβ-tubulin (46). We were unable to identify regions of CopN with tubulin-binding repeat-like sequence motifs and chose to test experimentally the idea that CopN might bind to a similar site on tubulin. We focused on the C-terminal plug domain (CopNΔ84) because longer constructs formed dimers in our gel filtration conditions, complicating the experiments. We evaluated the capacity of CopNΔ84 to bind stathmin-tubulin complexes and form a supershifted T2S-C complex (stathmin bound to two αβ-tubulin dimers bound to one or more CopN proteins). As shown in Fig. 4 and supplemental Fig. 4, CopN did not interact with the T2S complex, consistent with a CopN-tubulin interface that physically overlaps with the stathmin-tubulin interface. We verified this result with a competition binding experiment and determined that CopN competed with biotinylated stathmin for binding to immobilized tubulin (Fig. 4).
DISCUSSION

CopN is a multifunctional chlamydial effector protein functioning both as the T3SS plug protein and as a secreted effector protein that causes mitotic arrest (1, 4, 30, 34). To our knowledge, it is the only plug protein known to function also as an effector. As such, it is perhaps not surprising that the chaperone use of CopN is also unusual.

T3SS chaperones are typically grouped by substrate function, with class I chaperones binding effectors, class II binding translocators, and class III binding components of the needle complex (21, 47). The functional grouping into class I and class II chaperones corresponds with conserved structural characteristics. Class I chaperones are dimeric mixed α/β-proteins composed of five β-strands and three α-helices (21). Class II chaperones are monomeric all α-helical tetracopeptide repeat proteins (48–50).

We have shown that CopN binds directly to Scc3 (Fig. 1), in agreement with results obtained from yeast and bacterial two-
CopN Sequesters Tubulin and Prevents Microtubule Assembly

Regulation of MT assembly is a mechanism to control cell division utilized by both stathmin and CopN (4, 9, 57). Stathmin regulates MT assembly in a cell cycle-dependent manner, achieved by cell cycle-dependent phosphorylation of stathmin, which reduces its inhibitory effect and allows cytokinesis to proceed (9, 57). CopN also inhibits cell division (4), but two significant distinctions between these proteins, despite similar mechanisms, should be noted. Although stathmin is highly abundant (9) and its activity is regulated, allowing cell division (57), no such regulation of CopN is known. Furthermore, T3SS effectors are secreted in fairly small quantities; this has only been measured once, for SipA from Salmonella, where ~6000 molecules/bacterium are secreted (58), implying that similar biochemical functions are utilized in different ways to promote similar outcomes. CopN heterologous expression disrupts MTs and causes mitotic arrest (4), but it is unclear how many copies of CopN are required for this effect. It is known that, during infection, Chlamydia species do not disrupt the host cell cycle until the infections are fairly dense (17), which can be ~1000 bacteria/host cell (59–61). Thus, CopN could be present at very high levels in cells. Because αβ-tubulin is present at millimolar concentrations (~25 million molecules) (62, 63) in cells, CopN must either be abundant or targeted to the site of tubulin polymerization for αβ-tubulin sequestration to alter MT assembly significantly. Chlamydia species are indeed located at or near the MT-organizing center (18–20); thus, a mechanism involving tubulin sequestration at the site of tubulin polymerization seems likely. It remains to be determined if additional MT-disrupting functions can be attributed to CopN. From our data, we cannot rule out the possibility that CopN-bound αβ-tubulin might also be substoichiometrically incorporated into MTs, causing additional changes in MT dynamics.

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