Biochemical and Biophysical Research Communications **258,** 39–43 (1999)
Article ID bbrc.1999.0572, available online at http://www.idealibrary.com on IDEAL®

The Ncd Tail Domain Promotes Microtubule Assembly and Stability

A. Karabay and R. A. Walker[1]
Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received March 12, 1999

Non-claret disjunctional (Ncd) is a *Drosophila* kinesin-like motor required for spindle assembly and maintenance in oocytes and early embryos. Ncd has an ATP-independent microtubule binding site in the N-terminal tail domain as well as an ATP-dependent microtubule binding site in the C-terminal motor domain. The Ncd tail domain shares many properties with the microtubule-associated proteins that regulate microtubule assembly, including microtubule binding and bundling activity and an abundance of basic and proline residues. Given these similarities, we examined the ability of Ncd tail domain proteins to promote MT assembly and stability. The results indicate that the Ncd tail domain can promote MT assembly and stabilize MTs against conditions that induce MT disassembly, and suggest that Ncd may influence MT dynamics within the spindle. © 1999 Academic Press

Non-claret disjunctional (Ncd) is a *Drosophila* minus end-directed, kinesin-like motor protein required for spindle assembly and maintenance during meiosis in oocytes and early mitosis in embryos (1–6). The C-terminal motor domain (residues 356-700) contains an ATP-dependent microtubule (MT) binding site, and is attached to the N-terminal tail domain (residues 1-200) by an α-helical stalk domain responsible for subunit dimerization (1, 2). The Ncd tail (NT) domain sequence is unique among kinesin superfamily motors and is rich in basic and proline residues (1, 2, 7). Based on *in vitro* experiments in which Ncd bundled MTs (8), and NT proteins both bound and bundled MTs (7), it has been suggested that the Ncd tail contains an ATP-independent MT binding site (7, 8) that may aid in MT bundling and/or the sliding of a "cargo" MT along a "roadway" MT. *In vivo* analysis of Ncd mutants also supports the presence of an ATP-independent MT binding site in the tail of Ncd (3, 5). Recently, we have shown that Ncd residues 83-100 and 115-187 contain sites that exhibit ATP-independent MT binding with high and low affinity respectively, and have suggested that these MT interaction sites structurally combine in the native protein to form a single MT binding site (9).

The abundance of basic and proline residues in the NT domain is a common feature of many MT-associated proteins (MAPs), such as MAP2 and tau, that promote and stabilize MT assembly (see 10 and 11 for review). Given these similarities, we have examined the ability of the NT domain to promote microtubule assembly and to stabilize assembled MTs. Two different NT proteins were expressed as thioredoxin (Trx) fusions in *E. coli* and evaluated: TrxNT6, which corresponds to residues 83-187 and contains both the high and low affinity MT binding sites; and TrxNT8, which corresponds to residues 115-187 and contains only the low affinity MT binding site. MT sedimentation, turbidity measurements, video-enhanced differential interference contrast (VE-DIC) microscopy and electron microscopy (EM) were used to determine if TrxNT proteins promote MT assembly. MT sedimentation and turbidity measurements were also used to determine whether the TrxNT proteins stabilized MTs against conditions that induce MT depolymerization. We have found that TrxNT6 but not TrxNT8 can promote tubulin assembly into MTs and can stabilize MTs. These results suggest that the tail of Ncd, in addition to bundling spindle MTs (5–8), may also influence MT dynamics within spindles.

MATERIALS AND METHODS

Protein preparation. TrxNT6 and TrxNT8 were expressed in *E. coli* and purified as previously described (9). Tubulin was purified from porcine brain (12). Purified proteins were quick frozen in liquid nitrogen and stored at −70°C. Protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as a standard.

[1] Corresponding author. Fax: (540) 231-9307. E-mail: rawalker@vt.edu.

Abbreviations used: Ncd, non-claret disjunctional; MT(s), microtubule(s); TMT(s), taxol-stabilized microtubule(s); TrxNT, thioredoxin-Ncd tail fusion protein; MAP(s), microtubule associated protein(s); BSA, bovine serum albumin; EM, electron microscopy.

0006-291X/99 $30.00

MT sedimentation and turbidity assays. MT sedimentation assays were performed in AB buffer (20 mM Pipes, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA) containing 1 mM MgGTP and 100 μg/ml BSA. In a reaction volume of 100 μl, TrxNT6 or TrxNT8 (typically 5-35 μM final) was mixed with tubulin (5 μM final). After incubation for 15 minutes at 23°C, reactions were centrifuged at 100,000 × g for 15 minutes at 23°C. The resulting supernatants and pellets were then analyzed by SDS–PAGE (9). For MT stability assays, 50 μM tubulin was first polymerized at 37°C for 15 minutes and then diluted 10-fold into a reaction mixture containing TrxNT6 or TrxNT8. After 15 minutes at 23°C, identical reaction mixtures were either placed on ice, diluted a further 5-fold, or supplemented with either $CaCl_2$ (5 mM final) or NaCl (500 mM final). After 30 minutes, the reactions were centrifuged and processed as described above (except cold samples were centrifuged at 4°C).

MT assembly and stability were also evaluated by measuring turbidity at 350 nm in a Beckman DU-640 spectrophotometer. Tubulin (5 μM final) was mixed with TrxNT6 or TrxNT8 (1:1 TrxNT:tubulin), and A_{350} was measured for 30 minutes at 23°C. At 30 minutes, samples were either diluted 5-fold or supplemented with $CaCl_2$ (5 mM final), and A_{350} was measured for an additional 30 minutes.

Microscopy. Tubulin (5 μM final) was mixed with either TrxNT6 or TrxNT8 (2:1 or 4:1 TrxNT:tubulin), incubated for 15 minutes at 23°C, and observed with VE-DIC as described previously (12). Alternatively, the effect of TrxNT proteins on seeded MT assembly was examined in experiments in which tubulin was mixed with either TrxNT protein and immediately perfused into a slide-coverslip flow cell containing sea urchin (*Lytechinus pictus*) axonemes affixed to the glass surfaces (12).

For visualization by negative stain EM, tubulin (5 μM final) was mixed with either TrxNT6 or TrxNT8 (4:1 TrxNT:tubulin), incubated for 15 minutes at 23°C, adsorbed to carbon-coated 200 mesh grids, and stained with 2% uranyl acetate. In some cases taxol-stabilized MTs (TMTs) were substituted for tubulin. Stained samples were observed on a JEOL JEM-100CXII electron microscope.

RESULTS

To evaluate the ability of TrxNT6 or TrxNT8 to promote MT assembly, various concentrations of each TrxNT protein (5-35 μM final) were mixed with tubulin (5 μM final) and incubated at 23°C for 15 minutes. At this tubulin concentration and temperature, no MT nucleation or elongation is expected since 5 μM is well below the critical concentration for both nucleation and elongation of purified tubulin (12, 13). Samples were then subjected to centrifugation at 100,000 × g to pellet any polymer that formed, and supernatant and pellet fractions were analyzed by SDS–PAGE. In control reactions that contained tubulin, TrxNT6 or TrxNT8 individually, almost no protein was found in the pellet fraction (Figure 1a). However, when TrxNT6 was combined with tubulin, there was a concentration-dependent increase in the amount of tubulin (and TrxNT6) found in the pellet fraction as the TrxNT6:tubulin molar ratio was increased to 3:1. Above 3:1, the amount of tubulin and TrxNT6 in the pellet fraction remained constant (Figure 1b). In contrast, addition of TrxNT8 did not increase the amount of tubulin that pelleted even at a 7-fold molar excess of TrxNT8 over tubulin (Figure 1c). The ability of TrxNT6 but not

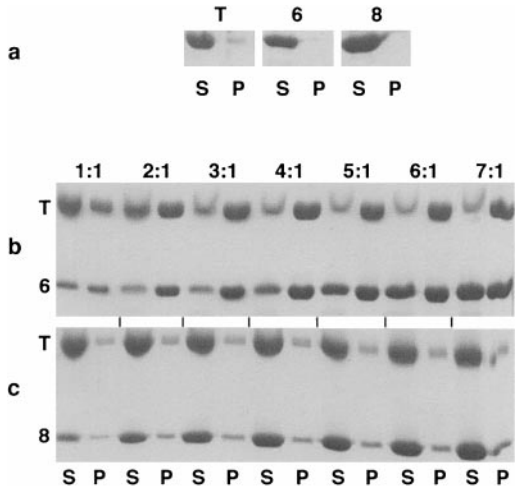

FIG. 1. Effects of TrxNT6 and TrxNT8 on tubulin assembly as measured by a sedimentation assay. MT sedimentation assays were performed as described under Material and Methods, and supernatant (S) and pellet (P) fractions were separated by SDS–PAGE and proteins stained with Coomassie Blue. Results for tubulin (T), TrxNT6 (6), and TrxNT8 (8) individually are shown in (a), and results for the indicated molar ratios of TrxNT6:tubulin and TrxNT8:tubulin are shown in (b) and (c) respectively.

TrxNT8 to promote tubulin assembly was confirmed by measurements of sample turbidity at A_{350} (Figure 2). Neither tubulin, TrxNT6, nor TrxNT8 produced a significant change in sample turbidity compared to the buffer only control. However, addition of TrxNT6 to tubulin caused a rapid and significant increase in turbidity (Figure 2a and c), while addition of TrxNT8 to tubulin had no effect (Figure 2b and d).

To examine the structures that pelleted in the sedimentation assay and caused turbidity at A_{350}, TrxNT-tubulin samples were observed by VE-DIC microscopy (Figure 3). TrxNT6 or TrxNT8 was mixed with tubulin (5 μM final) at 2:1 and 4:1 TrxNT:tubulin ratios and then observed after 15 minutes at 23°C. Consistent with the results of the sedimentation and turbidity experiments described above, no MTs were observed in reactions that contained only tubulin or tubulin and TrxNT8 (data not shown). However, TrxNT6 at both ratios induced polymerization of tubulin into MTs and generated bundling of the resulting MTs (Figure 3a and b). To determine if TrxNT6 or TrxNT8 promoted seeded tubulin assembly onto sea urchin axoneme fragments, each TrxNT protein was mixed with tubulin (4:1 TrxNT:tubulin ratio) and immediately perfused into a flow cell containing axonemes attached to the coverslip surface. No assembly onto axoneme ends was observed for samples that contained either tubulin alone or tubulin and TrxNT8 (data not shown). However, MT assembly was observed at axoneme ends for samples containing tubulin and TrxNT6 (Figure 3c), although these seeded MTs were subsequently ob-

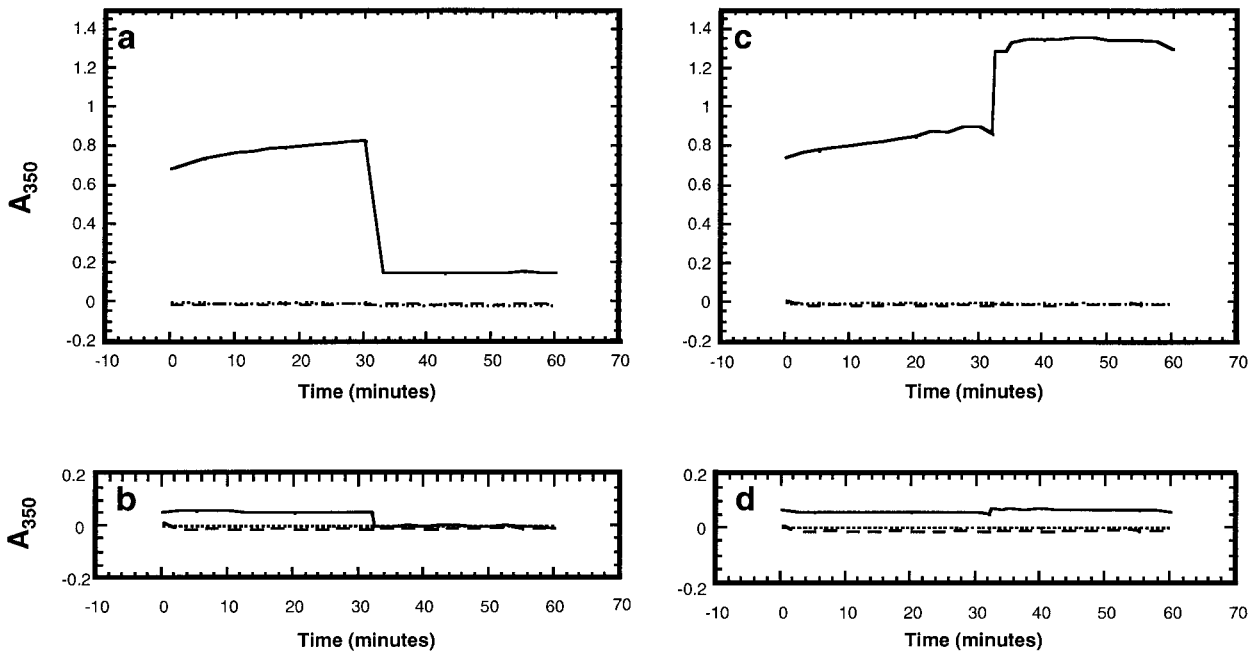

FIG. 2. Effects of TrxNT6 and TrxNT8 on tubulin assembly as measured by a turbidity assay. Tubulin (5 μM) was mixed with 5 μM TrxNT6 (a and c) or TrxNT8 (b and d), and A_{350} was measured for 30 minutes then samples were either diluted 5-fold (a and b) or supplemented with $CaCl_2$ to 5 mM (c and d), and A_{350} was measured for an additional 30 minutes. Tubulin only samples are indicated by the dotted line, TrxNT only samples are indicated by the dashed line, and TrxNT and tubulin samples are indicated by the solid line.

scured by multiple, short, thick MT bundles that assembled *de novo* (Figure 3d-f).

To obtain a higher resolution view of the polymers assembled in the presence of TrxNT6, TrxNT6-tubulin samples were observed by negative stain EM (Figure 4a). The polymers and bundles generated in samples containing 4:1 TrxNT6:tubulin appeared identical to those observed in samples of TrxNT6 and TMTs (Figure 4b). Further, the polymers present in the TrxNT6-tubulin samples were identical to polymers observed in samples of TMTs (Figure 4c), indicating that TrxNT6 promoted assembly of MTs rather than simply generating tubulin aggregates.

To evaluate the ability of TrxNT6 and TrxNT8 to stabilize MTs under conditions that induce MT disassembly, a modified MT sedimentation assay was used (Figure 5). In initial experiments, 50 μM tubulin was polymerized at 37°C to form MTs and then diluted 10-fold into AB buffer containing TrxNT6 or TrxNT8 (20 μM final to give a 4:1 TrxNT:tubulin molar ratio). After incubation at 23°C for 15 minutes, reaction mixtures were subjected to centrifugation to determine the ability of each TrxNT protein to stabilize MTs following dilution below the critical concentration for elongation. In the absence of TrxNT protein, tubulin was found in the supernatant fraction indicating that the MTs had disassembled following dilution (see Figure 1). Dilution of MTs into a solution containing TrxNT8 resulted in the majority of tubulin partitioning to the supernatant fraction, indicating that TrxNT8 did not stabilize MTs under these conditions (Figure 5). In comparison, dilution of MTs into a solution containing TrxNT6 resulted in the majority of tubulin partitioning to the pellet fraction, suggesting that TrxNT6 was able to stabilize MTs. To characterize further the MT stabilizing ability of TrxNT proteins, MTs were diluted into a solution containing TrxNT6 or TrxNT8 as described above, and then subsequently exposed to additional MT-destabilizing conditions (cold, further dilution to 1 μM tubulin, Ca^{++}) or to 500 mM NaCl for 30 minutes before centrifugation. Consistent with the failure of TrxNT8 to stabilize polymerized MTs against dilution to 5 μM tubulin, TrxNT8 had no stabilizing effect on MTs subjected to cold, dilution to 1 μM tubulin, Ca^{++}, or NaCl (data not shown). However, for MTs stabilized by TrxNT6, incubation on ice (Figure 5: cold), dilution to 1 μM tubulin (data not shown), or addition of $CaCl_2$ to 5 mM (Figure 5: $CaCl_2$) did not alter the relative distribution of tubulin into supernatant and pellet fractions, indicating that TrxNT6 also stabilized MTs against the effects of these disassembly-inducing conditions. Turbidity measurements of TrxNT6-tubulin samples either diluted 5-fold (Figure 2a) or supplemented with 5 mM $CaCl_2$ yielded results (Figure 2c) consistent with those observed in the sedimentation assay. Upon 5-fold dilution, sample turbidity also decreased $\approx$5-fold, while addition of $CaCl_2$ actually caused an increase in turbidity. The only agent that was effective at disassembling TrxNT6-MT complexes was 500 mM NaCl,

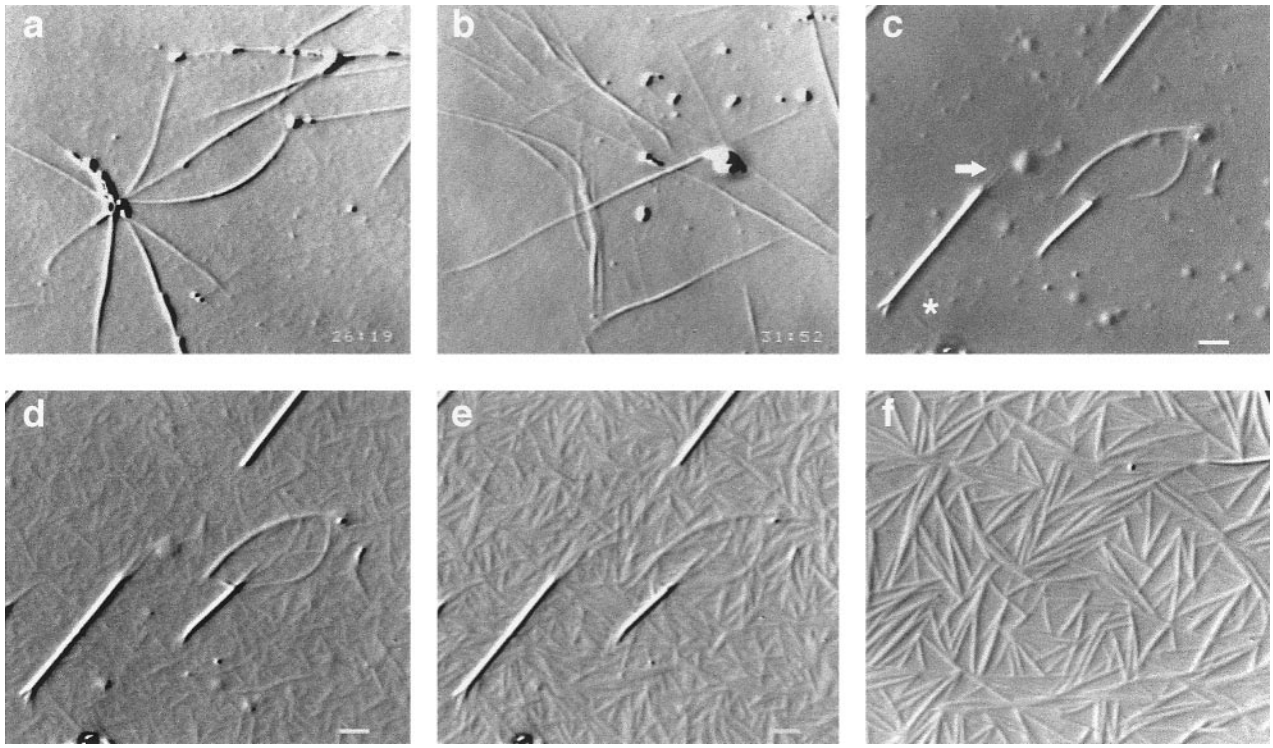

FIG. 3. TrxNT6-promoted MT assembly observed by VE-DIC microscopy. Samples were prepared to observe TrxNT6 promotion of MT self-assembly and seeded assembly as described under Materials and Methods. Two example fields of MTs and MT bundles resulting from TrxNT6-promoted MT self-assembly (TrxNT6:tubulin = 4:1) are shown (a, b). To determine effects on seeded assembly, a 4:1 TrxNT6:tubulin mixture was perfused into a flow cell containing axonemes. Assembly of MTs at ≈1 (c), 5 (d) and 15 (e) minutes post-perfusion is shown. The arrow in (c) indicates MTs assembling onto the end of an axoneme and the asterisk indicates a self-assembled MT. MT assembly in a different field at 20 minutes post-perfusion is also shown (f). The width of each image is equivalent to 25 μm.

which caused tubulin to redistribute to the supernatant fraction in the sedimentation assay (Figure 5).

DISCUSSION

Like tau and MAP2, the Ncd tail domain binds and bundles MTs and contains an abundance of basic and proline residues (1, 2, 10). These similarities suggest that the Ncd tail may be capable of influencing MT assembly. To address this possibility, two proteins that correspond to different portions of the Ncd tail domain were assayed for the ability to promote tubulin assembly and stabilize MTs. Both TrxNT6 (thioredoxin linked to Ncd residues 83-187) and TrxNT8 (thioredoxin linked to Ncd residues 115-187) have previously been shown to bind and bundle MTs (9). TrxNT6 contains two sites that interact with MTs, a high affinity site within residues 83-100 and a low affinity site within residues 115-187, while TrxNT8 contains only the low affinity site (9).

Promotion of tubulin assembly was analyzed by sedimentation and turbidity assays. In these assays, TrxNT8 did not increase the amount of tubulin that pelleted in the sedimentation assay (even at 7:1 molar excess over tubulin (Figure 1)) or the A_{350} value in the turbidity assay (Figure 2). In addition, when mixtures of TrxNT8 and tubulin were observed by VE-DIC or negative stain EM, no MTs were ever observed. Taken together, these results indicate that even though

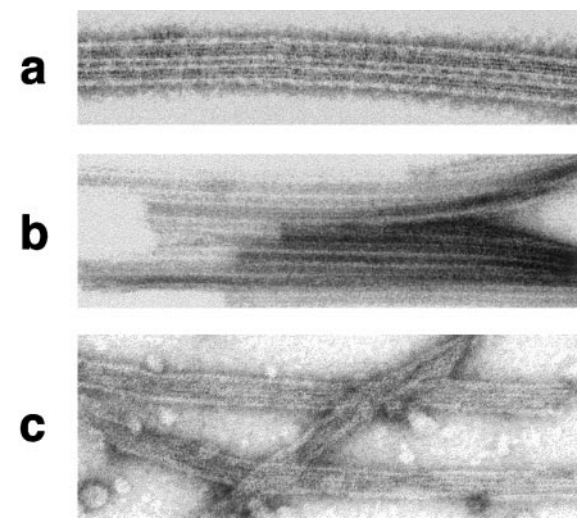

FIG. 4. EM images of TrxNT6 with tubulin and TMTs. Samples containing 4:1 TrxNT6:tubulin (a) or TrxNT6 and TMTs at a 4: 1 TrxNT6:tubulin ratio (b) were prepared and processed for negative stain EM as described under Materials and Methods. TMTs (c) are shown for comparison. Magnification ≈130,000.

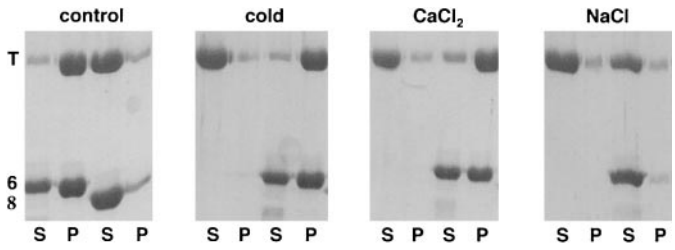

FIG. 5. The effects of TrxNT6 and TrxNT8 on MT stability. MT stability assays were performed as described under Material and Methods, and supernatant (S) and pellet (P) fractions were separated by SDS–PAGE and proteins stained with Coomassie Blue. The results for dilution of MTs to 5 μM tubulin in the presence of either TrxNT6 or TrxNT8 are shown (control), and the effects of additional MT destabilizing conditions (cold, $CaCl_2$, NaCl) on MTs (left 2 lanes) and TrxNT6-stabilized MTs (right 2 lanes) are also shown. The positions of tubulin (T), TrxNT6 (6), and TrxNT8 (8) are indicated.

TrxNT8 can bind TMTs (9), this protein, which contains only the low affinity MT interaction site, cannot promote tubulin assembly. Further, TrxNT8 was incapable of stabilizing MTs that were subjected to MT depolymerizing conditions such as dilution, cold, or Ca^{++} ions. In comparison, TrxNT6 increased the amount of tubulin that pelleted in the sedimentation assay as well as increasing sample turbidity at A_{350}. TrxNT6 was able to assemble tubulin at a 1:1 TrxNT6:tubulin ratio (Figure 1 and 2), and was maximally effective at a 3:1 ratio (Figure 1), which corresponds to the maximal binding stoichiometry of TrxNT6:tubulin (9). In addition to promoting tubulin assembly, TrxNT6 was able to stabilize MTs under conditions that induce MT depolymerization. In fact, $CaCl_2$ caused an unexpected increase in turbidity. The reason for this increase is unclear but may be due to increased MT bundling. The only condition that resulting in depolymerization of TrxNT6-stabilized MTs was 500 mM NaCl, which previously was shown to cause complete release of TrxNT6 from MTs (9). Although both the sedimentation and turbidity results support the hypothesis that TrxNT6 promotes tubulin assembly, neither assay provided information concerning the structures assembled. However, direct observation of the assembled products by VE-DIC and EM demonstrated that tubulin did in fact assemble into MTs in the presence of TrxNT6 (Figure 4).

The finding that TrxNT6 but not TrxNT8 promotes the assembly of tubulin into MTs and stabilizes MTs provides further evidence that the two previously identified MT-interaction sites in the Ncd tail (9) may combine to form a single MT binding site in the native protein, and suggests that the Ncd tail may be involved in regulation of MT assembly as well as attachment to a cargo MT. This is the first demonstration that the tail domain of a kinesin family motor may influence the dynamics of a cargo MT.

ACKNOWLEDGMENTS

This work was supported by an NIH grant (GM52340) (R.A.W.), a grant-in-aid of research from Sigma-Xi (A.K.), and a Graduate Research Development Project (GRDP) grant (A.K.) from the Graduate Student Assembly at Virginia Polytechnic Institute and State University. We are grateful to Bettina Deavours and Kali Phelps for their comments on this project and the manuscript.

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