The Mammalian Exocyst, a Complex Required for Exocytosis, Inhibits Tubulin Polymerization*

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The exocyst is a 734-kDa complex essential for development. Perturbation of its function results in early embryonic lethality. Extensive investigation has revealed that this complex participates in multiple biological processes, including protein synthesis and vesicle/protein targeting to the plasma membrane. In this article we report that the exocyst may also play a role in modulating microtubule dynamics. Using monoclonal antibodies, we observed that endogenous exocyst subunits co-localized with microtubules and mitotic spindles in normal rat kidney cells. To test for a functional relationship between the exocyst complex and microtubules, we established an in vitro exocyst reconstitution assay and studied exocyst effect on microtubule dynamics. We found that the exocyst complex reconstituted from eight recombinant exocyst subunits inhibited tubulin polymerization in vitro. Deletion of exocyst subunit sec5, sec6, sec15, or exo70 diminished its tubulin polymerization inhibition activity. Surprisingly, exocyst subunit exo70 itself was also capable of inhibiting tubulin polymerization, although exocyst complex with exo70 deletion did not lose its activity completely. Overexpression of exo70 in NRK cells resulted in microtubule network disruption and the formation of filopodia-like plasma membrane protrusions. The formation of these membrane protrusions was greatly hampered by stabilizing microtubules with taxol. Overexpression of exo84, an exocyst subunit that did not show tubulin polymerization inhibition activity, did not cause this phenotype. Results shown in this article, along with a previous report that localized microtubule instability induces plasma membrane addition, implicate a novel role for the exocyst in modulating microtubule dynamics underlying exocytosis.

The exocyst is a multisubunit complex first discovered for its requirement in the secretory pathway in yeast S. cerevisiae (1–3). Temperature-sensitive mutations in any of its eight subunits sec3, sec5, sec6, sec8, sec10, sec15, exo70, and exo84, resulted in defective secretion and polarized growth with concomitant accumulation of intracellular secretory vesicles under non-permissive temperature (1, 4). In mammals, the exocyst complex is also large complex similar to that found in yeast (5–9). The role of this complex in mammals and other multicellular organisms has been mostly studied through perturbation of its function in whole organisms and tissue culture cells. At the level of organism, knockout of exocyst subunits sec8 or sec10 resulted in early embryonic lethality (10–12). In tissue culture studies, inhibition of exocyst function, like in yeast, affected the secretory pathway. Perturbation of exocyst function in MDCK,1 pancreatic acinar, and NRK cells by an exo-exocyst subunit sec8 antibodies decreased protein targeting to various plasma membrane domains (13–15). Likewise, in the neuronal system, disruption of the exocyst function by exocyst subunit sec10 C-terminal deletion mutant overexpression and exocyst subunit sec5 knockout inhibited neurite outgrowth in neuroendocrine PC12 cells and cultured hippocampal neurons, respectively (11, 16). In addition, the exocyst subunit sec8 has been shown to play a role in targeting the NMDA glutamate neurotransmitter receptor to the plasma membrane via its association with the PDZ-binding protein SAP102 in cultured hippocampal neurons (17). Finally, the exocyst has also been shown to promote protein translation and filopodia formation (18–19). The involvement of exocyst in multiple cellular processes may explain, in part, the early lethality of exocyst mutants during development.

The indispensability of exocyst function during development has prompted extensive investigation into the molecular associations and biochemical mechanisms of its function. Currently, the exocyst has been found to associate, either directly or indirectly, with various cellular structures. These multiple associations may be partly responsible for the various functions associated with the exocyst complex in different cell types. In epithelial and mucosal LLC-PK1, MDCK, Caco-2 cell lines, exocyst subunits have been found to coimmunoprecipitate and co-migrate on density gradients with a subset of Golgi-derived vesicles and the plasma membrane fraction (13, 15, 20). In addition, in MDCK type II cells, exocyst subunit sec10 also coimmunoprecipitated with the sec61b component of the endoplasmic reticulum translocon (18). Finally, in neuroendocrine PC12 cells and cultured hippocampal neurons, we found exocyst subunits coimmunoprecipitated and co-migrated with tubulin (16). The localization of exocyst subunits in PC12 cells was dependent on microtubule integrity. These observations in

1 The abbreviations used are: MDCK, Madin-Darby canine kidney cells; GFP, green fluorescent protein; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; MOPS, 3-(N-morpholino)-propane-sulfonic acid; MAP, microtubule-associated protein; BSA, bovine serum albumin.

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PC12 cells led us to hypothesize that exocyst function may involve modulating microtubules. In this article, we tested this hypothesis by examining the in vivo co-localization of exocyst subunits with microtubules and investigating the effect of exocyst on microtubule polymerization dynamics.

EXPERIMENTAL PROCEDURES

Cell Culture—NRK cells were cultured at a density of 5,000 cells/cm² on culture dishes coated with 0.1 mg/ml polysine for transfection and immunocytochemistry experiments. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and grown at 37 °C in 5% CO₂.

For exocyst subunit overexpression studies, exo70 and exo84 cDNAs subcloned into GFP-C3 vector (Clontech; Palo Alto CA) were used. The resulting constructs were amplified in Escherichia coli bacteria and purified using Qiagen midi-prep kit (Qiagen; Valencia CA). These constructs were then transfected into NRK cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) as previously described for PC12 cells (16). The cells were transfected for 2 h. At 1 h following transfection, cells were treated with 0, 2, or 20 μM taxol for 18–24 h at 37 °C in 5% CO₂. The treated cells were then fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, washed with phosphate-buffered saline solution for 30 min and permeabilized with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at room temperature. The extent of tubulin polymerization was assessed by comparing against this 100% maximal tubulin polymerization for all our analyses. The extent of tubulin polymerization taking place in the presence of exocyst was assessed by comparing against this 100% maximal tubulin polymerization for all our analyses. The extent of tubulin polymerization was assessed by comparing against this 100% maximal tubulin polymerization for all our analyses.

Immunocytochemistry—Monoclonal antibodies against exocyst subunits sec8, exo84, and exo70 were generated using soluble recombinant exocyst subunits as described (16, 23). Polyclonal antibodies against tubulin were purchased from Cytoskeleton Inc. To monitor exocyst subcellular localization, NRK cells were fixed with methanol at room temperature or 37 °C for 1 min. We found that this fixation condition is optimal for preserving microtubule integrity and exocyst antibody reactivity in NRK cells. The fixed cells were rehydrated in phosphate-buffered saline solution for 30 min and permeabilized with Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum and 0.5% Triton X-100. Cells were washed three times with phosphate-buffered saline and incubated with primary antibodies overnight at 4 °C. Fluorescein-conjugated goat anti-mouse or rhodamine-conjugated donkey anti-sheep (Jackson Immunoresearch Laboratories, West Grove, PA) antibodies were then used as secondary antibodies. DNA was labeled by Hoechst dye (Sigma). Labeled cells were visualized by inverted fluorescence microscopy (Axiovert 200; Zeiss).

To monitor exocyst localization following nocodazole and cyclochalasin D treatment, NRK cells were incubated in the presence of 20 μM nocodazole or 10 μM cyclochalasin D for 1 h at 37 °C in 5% CO₂ before being subjected to immunostaining.

RESULTS

Exocyst Subunits Exhibit Filamentous Subcellular Distribution That Co-localizes with Microtubules and Mitotic Spindles—To begin investigating a functional relationship between the exocyst complex and microtubules, we studied the in vivo co-localization of endogenous exocyst subunits and microtubules in NRK cells. These cells are large and flat, and are thus ideal for intracellular protein localization studies. The localization of three endogenous exocyst subunits, sec8, exo70, and exo84, was detected by their respective monoclonal antibodies (Fig. 1A). Fig. 1, B, D, and G show that exocyst subunits sec8, exo70, and exo84 exhibited an apparent filamentous distribution. Co-staining of NRK cells with both anti-exocyst and anti-tubulin antibodies revealed that exocyst subunits co-localized with microtubules (Fig. 1, E, F, H, and I). In some cells, exocyst-containing filaments could be seen emanating with microtubules from a perinuclear region, possibly the microtubule-organizing center, toward the plasma membrane (Fig. 1, B, G, H, and I; arrows). This similar intracellular distribution of multiple exocyst subunits and their co-localization with microtubules bring up the possibility that the entire exocyst complex

formed was also collected at various time intervals after the initiation of polymerization by centrifugation at 18,000 × g in a Beckman microfuge R centrifuge. Pelleted microtubules were visualized by Coomassie Blue staining of SDS-polyacrylamide gels.

Negative Staining Transmission Electron Microscopy—To visualize microtubules following exocyst activity assay, the tubulin/exocyst mixture was adsorbed onto formvar/carbon-coated 200-mesh copper grids (EMS, Fort Washington, PA) for 1 min. The grids were then stained with 1% uranyl acetate for another minute and air-dried for 30 min before visualization at Rutgers electron microscopy facility. All procedures were carried out at room temperature. The extent of tubulin polymerization was assessed by quantitating the amount of microtubules in each electron micrograph using the Kodak one-dimensional image analysis program. The program reverses the dark/light contrasts in the micrographs (so microtubules would appear as bright filaments against a dark background) and estimates the relative quantity of microtubules in the micrograph by luminosity. Micrographs of blank electron microscopy grids subjected to the same negative staining procedure was used as the reference background luminosity. The final luminosity was determined by subtracting the reference background luminosity from the sample luminosity. The amount of tubulin polymerization occurred in the presence of equal protein concentration of BSA was assigned to be 100% maximal tubulin polymerization for all our analyses. The extent of tubulin polymerization taking place in the presence of exocyst was assessed by comparing against this 100% maximal tubulin polymerization for all our analyses.

Transmission electron microscopy. The amount of microtubules examined at 1 h after the initiation of tubulin polymerization by negative staining transmission electron microscopy. The amount of microtubules examined at 1 h after the initiation of tubulin polymerization by negative staining transmission electron microscopy. The amount of microtubules examined at 1 h after the initiation of tubulin polymerization by negative staining transmission electron microscopy. The amount of microtubules examined at 1 h after the initiation of tubulin polymerization by negative staining transmission electron microscopy.
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The observed exocyst co-localization with microtubules was not caused by fluorophore bleed-through because we still detected similar exocyst distribution in NRK cells stained with only anti-exo84 monoclonal antibodies (Fig. 1B). The staining pattern was also not likely an artifact of our fixation procedure. We have observed similar exocyst subunit localization with methanol fixation time ranging from 1–6 min (Data not shown). Using the same fixation condition, glyceraldehyde-3-phosphate dehydrogenase, a cytosolic glycolytic enzyme, displayed an expected diffuse cytosolic distribution (Fig. 1C). In addition, these monoclonal antibodies are specific for their respective antigens as assayed by Western blot, ELISA, and immunostaining of overexpressed exocyst subunits in NRK cells (Fig. 5A). Very little or no sec8, exo70, or exo84 staining was observed at the plasma membrane or Golgi apparatus as previously observed with other cell types. It is interesting to note that anti-exo70 and anti-exo84 antibodies used in this study localized these exocyst subunits to a perinuclear enrichment near or at the microtubule-organizing center (16) in PC12 cells. This difference in localization may be due to, at least in part, different exocyst association and/or regulation in various cell types (13, 15, 20). Alternatively, it is also possible that the anti-exo84 monoclonal antibodies we have used in this study recognize only a subpopulation of exocyst complex in the cell that associate with microtubules.

Finally, pharmacological studies, consistent with the above cell biological observations, showed that exocyst localization is dependent on microtubule, but not actin filament, integrity (Fig. 1, J–O). The filamentous distribution of both exo70 and microtubules was greatly disturbed by the microtubule-disrupting drug nocodazole (Fig. 1, J–L). Similar disruption was also observed with sec8 and exo84 (data not shown). The actin-disrupting drug cytochalasin D, on the other hand, caused actin filaments to aggregate into multiple foci but did not abolish the exocyst filamentous staining (Fig. 1, M–O). The slight distortion of exocyst distribution was most likely caused by the deformation of cytoplasm brought about by actin filament disruption. Taken together, the in vivo co-localization of exocyst subunits with microtubules, the dependence of their localization on microtubule integrity and the previous finding that exocyst and tubulin coimmunoprecipitated in vitro (16) bring up the possibility that the exocyst complex may play a role in modulating microtubule dynamics.

The Reconstituted Exocyst Complex Can Inhibit Tubulin Polymerization in Vitro—To investigate this hypothesis, we have established an in vitro assay to reconstitute the mammalian exocyst complex from eight recombinant exocyst subunits. Fig. 2A shows recombinant exocyst subunits: sec8, sec3, sec5, sec6, sec15, exo70, exo84, and sec10, purified from E. coli bacteria lysates (lanes sec8 to sec10). All subunits, with the exception of sec15, could be purified as intact proteins with molecular weights similar to that observed for rat brain exocyst subunits. The recombinant sec15 subunit was ~5 kDa smaller than the brain sec15 protein (data not shown). This subunit was very susceptible to degradation during the removal of GST tag from the GST-sec15 fusion protein by thrombin digestion.

To assess whether the recombinant exocyst subunits can associate to form a complex, purified exocyst subunits were mixed together in a 1:1 molar ratio and subjected to immunoprecipitation by anti-sec8 monoclonal antibody 822E12 (Fig. 2B, lane exo and exo(x-a-sec8)). This antibody has been shown to immunoprecipitate intact exocyst complex from brain lysate (6). It can also immunoprecipitate the recombinant sec8 alone (Fig. 2B, lane sec8). We found that the anti-sec8 antibody, either covalently cross-linked (lane exo(x-a-sec8)) or non-covalently bound (lane exo) to protein A beads, immuno-

Fig. 1. Exocyst subunits exhibit filamentous subcellular distribution that co-localize with microtubules and mitotic spindles in NRK cells. A, Western blots showing the specificity of anti-sec8 (lane 8), -exo84 (lane 84), and -exo70 (lane 70) monoclonal antibodies. These antibodies recognize a single protein band of the appropriate molecular weight in both PC12 (P) and NRK (N) cell lysates. The antibodies were used to localize exocyst subunits in NRK cells. Cells undergoing cell division at metaphase, anaphase, and telophase are denoted by arrowheads as M, A, and T, respectively. The enrichment of exocyst subunits near or at the microtubule organizing center is denoted by arrows. N denotes nucleus. B, immunofluorescence localization of sec8 (green). DNA was stained by Hoechst dye (blue). C, immunofluorescence localization of the cytosolic marker, glyceraldehyde-3-phosphate dehydrogenase. D–F, immunofluorescence micrographs showing the intracellular distribution of exo70 (D), microtubule (E), and their co-localization (F). The inset in D shows an enlarged picture of exo70 staining at metaphase. G–I, immunofluorescence micrographs showing the intracellular distribution of exo84 (G), microtubule (H), and their co-localization (I). J–L, localization of exo70 (J), microtubule (K), and their overlay (L) following treatment of NRK cells with nocodazole. M–O, localization of exo70 (M), actin (N), and their overlay (O) following treatment of NRK cells with cytochalasin D.
precipitated all eight exocyst subunits (Figs. 2B and 3A). This immunoprecipitation was specific because nonspecific immunoglobulin did not immunoprecipitate any exocyst subunit (Fig. 2B, IgG). In addition, the anti-sec8 antibody did not immunoprecipitate other exocyst subunits in the absence of sec8 subunit (Fig. 2B, exo-sec8). The coimmunoprecipitation of eight exocyst subunits by anti-sec8 antibodies indicates that reconstituted exocyst subunits can form the exocyst complex in vitro.

Once we have reconstituted the exocyst complex, we tested its effect on tubulin polymerization. We incubated the reconstituted exocyst complex with MAP-rich tubulin preparation and examined microtubule formation by negative staining transmission electron microscopy. We used microscopy instead of spectrophotometry as our initial microtubule detection method because it allowed us both to perform experiments using minimal amounts of reconstituted exocyst complex and to ensure that tubulin forms microtubules rather than amorphous protein aggregates under our assay conditions. The limiting factor in this assay was the availability of high concentrations of soluble exocyst subunits. While the exocyst subunits preferred high pH and salt conditions for maximal solubility, tubulin polymerization required lower pH and salt concentration. Therefore, the pH and salt concentration used in these assays were empirically determined to find an optimally compromised condition to achieve highest possible tubulin polymerization and exocyst subunit solubility.

We routinely carry out tubulin polymerization at a MAP-tubulin concentration of 13.5 μM because it is the minimal tubulin concentration at which we can still achieve robust and reproducible polymerization. When we incubated tubulin with the reconstituted exocyst complex at tubulin to exocyst molar ratios of 45:1 (0.3 μM exocyst), 68:1 (0.2 μM exocyst), and 135:1 (0.1 μM exocyst), we found that microtubule formation was inhibited by 90, 80, and 50% respectively (Fig. 2, C and D). We could not use lower tubulin to exocyst ratios in our assays because of limited solubility of the reconstituted exocyst complex. The inhibition of microtubule formation in the presence of reconstituted exocyst complex was not due to GTP depletion by the exocyst complex since increasing GTP concentration in our assay did not increase the amount of microtubules formed (data not shown). In addition, the inhibition of tubulin polymerization by the exocyst complex was also not a result of buffer or protein introduction to microtubules. Incubation of microtubules with BSA (Fig. 2C) at the same protein concentration or with exocyst purification buffer alone did not prevent tubulin polymerization. This observed tubulin polymerization inhibition activity by the exocyst complex is similar to that observed for stathmin which has been shown to inhibit tubulin polymerization at tubulin/stathmin ratios of 14:1 or 70:1 (24). However, unlike stathmin, which is a small 18 kDa protein, the exocyst is a 734 kDa complex composed of eight subunits. These observations raise the question of whether the tubulin polymerization inhibition activity of the exocyst complex is exclusive to a single exocyst subunit or requires cooperation among multiple exocyst subunits.

Exocyst Subunits Have Differential Effects on Tubulin Polymerization—To address this question, we investigated the extent of tubulin polymerization in the presence of reconstituted exocyst complexes with single subunit omissions. The formation of these complexes was concomitantly assessed by immunoprecipitation with anti-sec8 antibody. Fig. 3A shows that the omission of individual exocyst subunits, except in the case of sec8 omission (Fig. 3A, lane 8) in which we could not immunoprecipitate the exocyst complex in the absence of sec8, did not inhibit the coimmunoprecipitation of the remaining seven subunits. This observation suggests that seven out of
Exocyst subunits have differential effects on tubulin polymerization. Exocyst complexes with single subunit omission were reconstituted by mixing seven exocyst subunits in a 1:1 molar ratio. The formation of the complex was tested by immunoprecipitation with anti-sec8 antibody. The precipitated complex was visualized by SDS-PAGE and Coomassie Blue staining. The asterisk at the left side of each lane denotes the position of each missing subunit. The corresponding missing subunit was noted above each lane. B–D, sample electron micrographs of three classes of activity exhibited by the single subunit-omission exocyst complexes. The effect of exocyst complexes with single subunit omissions on tubulin polymerization was examined by adding 0.3 μM of reconstituted complex to 13.5 μM MAP-rich tubulin. The amounts of microtubules formed were assessed by negative staining electron microscopy. Single subunit deletion complexes showing the presented activity were indicated above each micrograph. Bar = 10 μm. E, graph of tubulin polymerization inhibition by exocyst complexes. BSA and exo denote bovine serum albumin and intact exocyst complex, respectively. Error bar represents S.D. in each category. n is five independent experiments.

Eight exocyst subunits could still associate with each other to form a complex in vitro. The observed coimmunoprecipitations were specific because nonspecific immunoglobulin did not immunoprecipitate the exocyst subunits (lane Ig). In addition, the anti-sec8 antibody did not immunoprecipitate other exocyst subunits in the absence of sec8 subunit (lane 8). These immunoprecipitation results are consistent with previous in vitro protein binding and yeast two-hybrid studies, which showed that each exocyst subunit can interact with multiple exocyst subunits (4, 8, 16). The omission of a single exocyst subunit, therefore, would not affect the assembly of other seven subunits into the complex in vitro. However, it is not clear whether the overall structure of the exocyst complex changes with single subunit omission.

When these single subunit omission exocyst complexes were tested for their ability to inhibit tubulin polymerization, three major classes of activities were observed (Fig. 3, B–D). Exocyst complexes with sec8, sec3, sec10, or exo84 deletion (Fig. 3B, -8, -3, -10, -84, and E) did not show significant decreases in their tubulin polymerization inhibition activity compared with the intact exocyst (Fig. 3E, exo). Approximately 80–92% of maximal tubulin polymerization was still inhibited in the presence of these complexes (Fig. 3E). Exocyst complexes without sec6, and exo70 (Fig. 3, C, -6, -70 and E), on the other hand, showed moderate activity, inhibiting ~50% of maximal tubulin polymerization. Finally, exocyst complexes without sec5 and sec15 (Fig. 3D, -5, -15 and E) lost most of their activity, inhibiting only 10–20% of maximal tubulin polymerization. These results suggest that multiple exocyst subunits participate in inhibiting tubulin polymerization. The loss of tubulin polymerization inhibition activity associated with exocyst subunit sec5, sec6, sec15, and exo70 omission may be due to their involvement either in tubulin and/or MAP binding, or in functional complex conformation maintenance.

Exocyst Subunit exo70 Alone Can Inhibit Tubulin Polymerization—The perturbation of exocyst activity by single subunit omissions brings up the possibility that individual exocyst subunits may also be capable of inhibiting microtubule formation. To investigate this possibility and to identify the exocyst subunit that can inhibit tubulin polymerization, we incubated tubulin with individual purified recombinant exocyst subunits (Fig. 4). Of the eight exocyst subunits, only Exo70 (Fig. 4C, 70) showed significant tubulin polymerization inhibition activity. Its activity was comparable to that observed with the intact exocyst complex of the same molar concentration (Fig. 4D). Sec15 (Fig. 4, B and D) also exhibited minor tubulin polymerization inhibition activity (inhibited ~25% of maximal tubulin polymerization). The remaining subunits (Fig. 4, A and D) had no significant activity. Thus, although exocyst subunits sec5 and sec6 are required for the tubulin polymerization inhibition activity of the intact exocyst complex, they do not have activity on their own. It is also interesting to note that even though exo70 by itself has tubulin polymerization inhibition activity comparable to that of the intact exocyst complex of the same molar concentration, deletion of exo70 does not completely abolish the exocyst activity. These results suggest that multiple exocyst subunits may cooperate to achieve optimal tubulin polymerization inhibition even though exo70 alone is sufficient to inhibit tubulin polymerization.

To ensure that the inhibition of tubulin polymerization by exo70 was not due to the presence of contaminating exocyst subunits in the MAP-enriched tubulin preparation, exo70 activity was also examined using purified tubulin (Fig. 5). This tubulin preparation did not contain exocyst subunits detectable...
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Fig. 4. Exocyst subunit exo70 alone can inhibit MAP-promoted tubulin polymerization. A–C, sample electron micrographs of three classes of activity exhibited by individual exocyst subunits. The effect of individual exocyst subunits on tubulin polymerization was carried out by incubating 0.3 μM of individual exocyst subunits with 13.5 μM MAP-rich tubulin. The amounts of microtubules formed were assessed by negative staining electron microscopy. Individual subunits showing the presented activity were indicated above each micrograph. We often observed black precipitates in tubulin samples that did not polymerize (C, arrow). These precipitates may represent tubulin aggregates that failed to polymerize. Bar = 10 μm. D, graph of tubulin polymerization inhibition by individual exocyst subunits. BSA and exo denote bovine serum albumin and intact exocyst complex, respectively. Error bar represents S.D. in each category. n = five independent experiments.

Fig. 5. Effect of exo70 on polymerization of purified tubulin. 25 μM purified tubulin was allowed to polymerized in the presence of 1 μM exo70 or of a control protein, exocyst subunit exo84. In A, the time course of tubulin polymerization in the presence of exo70 (open circles) or of exo84 (filled square) was monitored by optical scattering at 340 nm. Error bar represents S.D. in each category. n = four assays. In B, tubulin samples collected at 20 min after polymerization in the presence of exo70 (+exo70) or exo84 (+exo84) were examined by electron microscopy at two magnifications. Bar in panel i = 1 μm for both panels i and iii. Bar in panel ii = 0.1 μm for both panels ii and iv. In C, microtubules formed in the presence of exo70 (+exo70) and exo84 (+exo84) at 0, 20, and 90 min intervals were harvested by centrifugation. Pelleted microtubules were fractionated by SDS-PAGE and visualized by Coomassie Blue staining.

by Western blotting (data not shown). The effect of exo70 on tubulin polymerization was monitored by three assay methods: optical scattering at 340 nm, electron microscopy, and SDS-PAGE of microtubules harvested by centrifugation. The time course of tubulin polymerization in the presence of exo70, or of the control exocyst subunit exo84, was monitored by optical scattering at 340 nm. The presence of exo70 slowed the tubulin polymerization process and decreased the amount of polymerized tubulin during the 90-min assay period (Fig. 5A). Samples of microtubules formed during the assay period were also examined by negative staining transmission electron microscopy (Fig. 5B). Using this assay method, it was also obvious that there were fewer microtubules formed in the presence of exo70 than exo84. However, no obvious differences in microtubule length (Fig. 5B, panels i and iii) and structure of microtubule ends (Fig. 5B, panels ii and iv) were observed between microtubules formed in the presence of exo70 and exo84. Finally, the amount of microtubules formed at various time intervals during the polymerization study was also collected by centrifugation and visualized by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 5C). As expected, more microtubules were harvested from tubulin samples incubated with exo84 (+exo84) than with exo70 (+exo70). Using the Kodak one-dimensional image analysis software to assess the staining intensity of the tubulin protein bands on the gel, it was estimated that there was ~50% less microtubules formed in the presence of exo70 than in the presence of exo84 at 90 min after the initiation of tubulin polymerization. These results strongly suggest that exo70 alone can inhibit tubulin polymerization in vitro. In addition, the above observations also imply that exocyst may play a role in destabilizing microtubule in vivo.

Overexpression of Exocyst Subunit exo70, but Not of exo84, Perturbs the Microtubule Network in the Cell and Promotes the Formation of Plasma Membrane Protrusions—From the above in vitro results we hypothesize that the overexpression of exocyst subunit exo70 in the cell may promote microtubule end instability. One potential consequence of this event is an increased plasma membrane addition. Zakharenko and Popov (25) have previously reported that a localized microtubule destabilization by the microtubule-disrupting drug nocodazole facilitated, and in some cases was sufficient to promote, local plasma membrane addition. The addition of the microtubule-stabilizing drug taxol, on the other hand, inhibited plasma membrane addition. Based on these findings, we hypothesize that the overexpression of exo70 should result in increased membrane addition, a process that can be blocked by stabilizing microtubules with taxol. To test this hypothesis, we overexpressed GFP-exo70 in NRK cells by transient transfection. As a control, we also overexpressed GFP-exo84, an exocyst subunit that does not affect tubulin polymerization. It is interesting to note that the overexpressed GFP-exo70 and GFP-exo84 exhibited an overall diffuse localization throughout the cell, as assessed by GFP fluorescence (Fig. 6A, GFP-exo70 and GFP-exo84) and by monoclonal antibody staining (α-exo70 and α-exo84). The antibody staining is not an artifact of fluorophore bleed-through because untagged overexpressed exo70 and exo84 also exhibited the same diffuse localization (data not shown). This observation is consistent with previous findings in MDCK cells in which GFP-tagged exocyst subunits showed diffuse intracellular localization (9). In addition, GFP-exo84 also showed a moderate enrichment in the nucleus. This nuclear enrichment of GFP-exo84 may be due to its activity in pre-mRNA splicing as previously reported in yeast (26). These overexpressed and GFP-tagged proteins did not have the same restricted localization as that observed for endogenous exocyst
subunits, suggesting that they may not be able to incorporate into the endogenous exocyst complex.

Fig. 6B shows that many NRK cells with overexpressed GFP-exo70 seemed to have increased plasma membrane surface area in the form of long, thin, and sometimes highly branched plasma membrane protrusions (panel 70, arrow). To assess the frequency of GFP-exo70- and GFP-exo84-transfected NRK cells having excessive plasma membrane protrusions, we counted cells that had at least 20 plasma membrane protrusions with length equal or greater than one-twentieth of the cell diameter. Approximately 75% of NRK cells transfected with GFP-exo70 developed plasma membrane protrusions that satisfied these criteria (Fig. 6C). In the presence of microtubule-stabilizing drug taxol, however, only 30% of cells developed plasma membrane protrusions in sufficient number and length to be included in our cell count. Many cells had only very short plasma membrane spikes (Fig. 6B, panel 70T, arrow). To ensure that the decreased cell count associated with taxol treatment was not due to taxol-induced cell death, NRK cells were treated with two concentrations of taxol. We did not find evidence that this treatment was not due to taxol-induced cell death, NRK cells transfected with GFP-exo70 and treated with 2 and 20 μM taxol, respectively. 84 + 2T denotes cells transfected with GFP-exo84 and treated with 2 μM taxol. Error bar represents S.D. in each category. n is 200 for each category.

DISCUSSION

The exocyst complex is present in all cell types and organisms examined so far, suggesting that its function is ubiquitously required. In addition, the exocyst has also been shown to play a role in multiple cellular processes such as Golgi-to-plasma membrane vesicle/protein trafficking and protein synthesis. Understanding the molecular mechanisms underlying its function should provide important insights into these biological processes. In this article, we present evidence suggesting that a mechanism underlying the exocyst function involves modulating microtubule dynamics. Three lines of evidence have led us to hypothesize a functional relationship between exocyst and microtubules. First, although all exocyst subunits are soluble proteins, the majority of exocyst complex in rat brain appeared to be present in an insoluble fraction (6). Density gradient centrifugation and immunoprecipitation studies showed that exocyst subunits coimmunoprecipitated with tubulin (16), suggesting that the insolubility of exocyst in brain may be due to, in part, its association with microtubules. These observations are consistent with the presence of exocyst sub-
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units exo70, exo84, sec6, and sec8 in commercially available MAP-rich tubulin preparations from Cytoskeletons Inc. (data not shown). The co-purification of exocyst subunits with tubulin through repeated tubulin polymerization and depolymerization cycles suggests that various exocyst subunits or the exocyst complex can associate with tubulin or tubulin-associated proteins. Second, immunofluorescence studies show that endogenous exocyst subunits co-localize with microtubules. The co-localization of exocyst with microtubules on mitotic spindles in dividing NRK cells indicates that exocyst can associate, either directly or indirectly, with microtubules without secretory vesicles. It is interesting to note that while exocyst co-localizes with the majority if not all microtubules in normal rat kidney cells, it only co-localizes with a subset of microtubules in PC12 cells (data not shown). It is possible that the exocyst either associates specifically or is enriched with a subset of microtubules involved in polarized growth, such as during neurite outgrowth, in highly polarized cells. Third, despite whether exocyst associates with most or a subset of microtubules, microtubule integrity is essential for proper exocyst localization, as microtubule-disrupting drugs drastically alter exocyst localization. Taken together, these observations strongly suggest a physical and possibly a functional association between the exocyst and microtubules. The question now is: how are microtubules involved in exocyst function?

To begin addressing this question, we have established an in vitro exocyst reconstitution assay in which we can assess the effects of intact and partial exocyst complexes, as well as of individual exocyst subunits, on microtubule dynamics. Using this assay system, we found that the intact exocyst complex, as well as the exo70 subunit alone, can inhibit tubulin polymerization in vitro. These findings, along with a previous report that localized destabilization of microtubules can promote plasma membrane addition (25), led us to hypothesize that exo70 overexpression may disrupt microtubule network and increase plasma membrane addition. Indeed, we observed that the overexpression of exo70 resulted in localized disruption of microtubule network in the cell and increased plasma membrane addition in the form of long and thin plasma membrane protrusions. The formation of these exo70-induced microtubule protrusions was greatly inhibited by stabilizing microtubules with taxol. These in vitro and in vivo observations are consistent with a role for exo70, and thus exocyst, in modulating microtubule dynamics underlying plasma membrane addition.

In this regard, it is interesting to note that in neuroendocrine PC12 cells the exocyst has a similar intracellular localization as that of the microtubule-destabilizing protein SCG10 (24). Both macromolecules are enriched in the growth cone where high levels of microtubule ends are present. The exocyst complex may function in coordination with stathmin to promote localized microtubule end instability, leading to plasma membrane addition at the growth cone. In agreement, SCG10 overexpression has also been shown to enhance neurite outgrowth in PC12 cells (24). Currently, the molecular mechanisms underlying this localized microtubule destabilization and plasma membrane addition are not known. Further understanding of the exocyst association with tubulin and/or microtubule-associated proteins should provide valuable insights into the functional relationship between exocyst and microtubules and how microtubule dynamics can affect exocytosis.

In conclusion, the exocyst, a large complex composed of eight subunits, has been reported to associate with various cellular structures such Golgi-derived secretory vesicles, plasma membrane and endoplasmic reticulum. These multiple associations have led to the proposal of several models on the mechanisms of this complex in promoting protein synthesis and vesicle trafficking. In particular, the exocyst complex is best known for its involvement in protein-vesicle targeting to the plasma membrane. In yeast, mutations in exocyst subunits resulted in the accumulation of secretory vesicles near the plasma membrane (1, 4). This observation, along with in vitro association of exocyst with secretory vesicles and plasma membrane, has led to the proposal of exocyst acting as a tethering protein to mediate vesicle docking and fusion at the plasma membrane during exocytosis. In this article we propose that, in addition to its role as a tethering protein, the exocyst may also participate in regulating microtubule dynamics. Presently, the process of transferring Golgi-derived vesicle from microtubule ends to actin and finally to the plasma membrane is poorly understood. The exocyst complex, with its eight subunits, may participate in more than one event in this process to promote plasma membrane addition. Thus, the multiple, and likely regulated, association of exocyst with various cellular structures may play a role in coordinating protein synthesis, vesicle trafficking and cytoskeletal organization to promote cell growth and cell differentiation. Furthermore, some potential exocyst regulators discovered to date include members of the small GTPase family such as Rho, Ral, Rab, and TC10 which are downstream messengers of many signaling pathways (8, 19, 27–31). The establishment of in vitro exocyst reconstitution assay and the availability of exocyst subunit-specific monoclonal antibodies should allow us to further investigate the regulation of exocyst function in response to various intracellular and extracellular signals during development.

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