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Role of Gamma-synuclein in Microtubule Regulation

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Abstract

Gamma-synuclein is a neuronal protein found in peripheral and motor nerve systems. It becomes highly expressed in metastatic but not in primary tumor or normal tissues. The close association between gamma-synuclein expression and cancer spreading has been demonstrated in a broad range of malignancies. Our previous study showed that exogenous expression of gamma-synuclein in ovarian and breast cancer cells significantly enhanced cell migration and resistance to paclitaxel-induced apoptotic death. In our current research, we found that gamma-synuclein can affect microtubule properties and act as a functional microtubule associated protein. *In vitro* assays revealed that gamma-synuclein can bind and promote tubulin polymerization, induce the microtubule bundling and alter microtubules morphology developed in the presence of microtubule associated protein 2 (MAP2). Using cancer cell lysate, gamma-synuclein protein was found to be localized in both cytosolic compartment and extracted cytoskeleton portion. Immunofluorescence staining demonstrated that gamma-synuclein can colocalize with microtubule in HeLa cells and decrease rigidity of microtubule bundles caused by paclitaxel. In human ovarian cancer epithelial A2780 cells, gamma-synuclein overexpression improved cell adhesion and microtubule structure upon paclitaxel treatment. Importantly, it led to microtubule-dependent mitochondria clustering at perinuclear area. These observations suggest that overexpression of gamma-synuclein may reduce cell chemo-sensitivity of tumor cells through decreasing microtubule rigidity. In summary, our studies suggested that gamma-synuclein can directly participate in microtubule regulation.

Introduction

Synuclein family consists of three small acidic neuron proteins: alpha-, beta- and gamma-synuclein. While alpha- and beta-synucleins are predominantly expressed in central nerve system, gamma-synuclein is mainly detected in peripheral and sensory neurons. Direct implications of alpha-synuclein in Alzheimer and Parkinson Disease have been well documented. It accumulates in the Lewy bodies and Lewy neurites and forms toxic fibrils. Beta- and gamma-synucleins have also been implicated in hippocampal axon pathology in

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Parkinson's disease [1]. Specific changes of gamma-synuclein expression in retina and optical nerve were reported in Alzheimer's disease patients [2].

In addition to their pathological role in neurodegenerative diseases, accumulating evidence suggest that synucleins, especially gamma-synuclein, may contribute to cancer metastasis. Originally discovered as BCSG1 (Breast Cancer Specific Gene1), gamma-synuclein detected in tumor was found to correlate with metastatic status in a broad spectrum of malignancies including pancreatic, esophagus, colon, gastric, lung, prostate, cervical, and breast cancer [3-5]. Stage-specific expression of gamma-synuclein was detected in various cancer types at the pattern of very low expression in stage I but high expression in stages II to IV. Patients bearing gamma-synuclein-expressing tumors had a significantly shorter DFS (disease free survival) and a high probability of death when compared with those without gamma-synuclein tumor expression [6]. Like gamma-synuclein, upregulation of alpha- and beta-synuclein was also reported in a high percentage of ovarian and breast carcinomas [7]. These discoveries suggested that synucleins, especially gamma-synuclein, may be a significant contributor to cancer development and progression.

Microtubules are bundles of protofilaments formed by polymerized alpha- and beta-tubulin dimers. As a major component of cytoskeleton network, they are critical in the maintenance of cell shape and polarity, mitosis, cytokinesis, cell signaling, as well as intracellular trafficking (e.g. vesicular and mitochondria transport). Its assembly, organization and dynamics were precisely regulated through multiple means including the interaction with microtubule associated proteins and serving proteins, posttranslational regulations and differential expression of specific isoforms. In cancer cells, elevations in the level of certain tubulin isoforms as well as microtubule associated proteins can directly affect chemo-resistance [11-13].

Paclitaxel is one of most widely used chemotherapeutic agent in cancer treatment. It induces cancer cell death through overstabilizing microtubule networks and disrupting microtubule-mediated cellular events. Our previous studies show that overexpression of gamma-synuclein in breast and ovarian cancer cells significantly decreased paclitaxel-induced apoptotic death, which was further confirmed by Zhou Y et al using siRNA strategy [14]. In both studies, gamma-synuclein was found to reduce cytotoxicity mediated by anti-microtubule but not DNA repair reagents. These findings suggested that gamma-synuclein may protect cancer cells from paclitaxel via a microtubule-specific mechanism rather than altering drug efflux pathways. To test this hypothesis, we examined the effect of synucleins on microtubule properties *in vitro* and *in vivo*. We found that gamma-synuclein can bind to microtubules, affect their stability, induce microtubule bundling and co-regulate microtubule phenotype with MAP2. In addition, our results indicated that gamma-synuclein may also regulate microtubule morphology and microtubule-dependent mitochondria trafficking in cancer cells.

Materials and Methods

Materials

Paclitaxel was purchased from Sigma. Anti-gamma-synuclein antibodies were purchased from Santa Cruz Inc., anti-beta tubulin antibodies from Cytoskeleton Inc, and anti-beta actin antibodies were from Sigma. Alexa568-anti-goat secondary antibody was purchased from Invitrogen. Human recombinant gamma-synuclein was purchased from Alpha Diagnostic International. Human recombinant MAP2 and kinesin heavy chain motor domain protein were purchased from Cytoskeleton Inc. MitoTracker Red was purchased from Invitrogen.

Cell culture

Breast cancer cell line T47D and GFP-tubulin HeLa cell line (kindly provide by Dr. Tim Yen at Fox Chase Cancer) that stably expressing GFP tagged tubulin were cultured in DMEM supplemented with 10% FBS, L-glutamine and P/S. A2780 parental cells and A2780 cells stably overexpressing human gamma-synuclein were established as previously described [15] and maintained in RPMI supplemented with 10% FBS, L-glutamine, P/S and insulin.

Transfection

The method for stably transfecting human gamma-synuclein gene into A2780 cells was described previously [15]. For transient transfection of human gamma-synuclein or empty control vector into GFP-tubulin HeLa cells, Lipofectamine2000 (Invitrogen) was used and the protocols provided by the company were followed. Briefly, GFP-tubulin HeLa cells were cultured to approximately 80~90% confluency before transfection. pCDNA3-gamma-synuclein and empty control vector were mixed with lipid in Opti-MEM at recommended ratios and incubated for 30 min at room temperature to form DNA-lipid complex. DNA-lipid complexes were added to cell culture and incubated overnight. The transfection efficiency was examined either by immunofluorescence staining or western blot analysis.

Immunofluorescence staining and microscopy

To visually assess the co-localization between GFP microtubules and gamma-synuclein, GFP-tubulin HeLa cells with transient gamma-synuclein expression were cultured on two-well or four-well chamber slides at approximately 60~70% confluency. To preserve the microtubule structure, cells were treated with microtubule stabilization buffer (MTSB, 4 M glycerol, 100 mM PIPES pH 6.9, 1 mM EGTA) for 1 min followed by 2 min in MTSB + 0.5% TritonX-100 and immediate fixation in 3.7% paraformaldehyde for 5-7 min. For gamma-synuclein staining, the slides were washed with PBS three times and blocked with 3% BSA. The slides were then washed with PBS and incubated at room temperature with goat-anti-human-gamma-synuclein antibody diluted in 3% BSA (1:100). 1 hr later the slides were washed with PBS three times again and incubated with Alexa568-anti-goat secondary antibody for 1 hr. The slides were washed three times with PBS and mounted with Vectashield containing DAPI. For mitochondria staining, A2780 cells seeded in two-well or four-well chamber slides were incubated with MitoTracker for 15 min at room temperature. Then slides were washed with PBS and fixed with methanol for 5 min. The slides were washed again and subjected to mounting and sealing. For Immunofluorescence microscopy, image was captured (600×) either using Nikon ECLIPSE TE300 microscope or Nikon E800 upright microscope with BioRad Radiance 2000 confocal scanhead. LaserSharp2000 software was used for confocal image acquisition and procession.

Tubulin polymerization assay

Tubulin polymerization assay was performed using Tubulin Polymerization Assay Kit (Cytoskeleton, Inc.) and followed the protocols provided by the manufacturer. Briefly, 200 µl of highly purified tubulin from bovine brain at 20 µg/µl concentration was mixed with 420 µl ice cold tubulin polymerization buffer to give a final concentration of 3 mg/ml tubulin in 8 mM PIPES pH6.9, 2 mM MgCl₂, 0.5 mM EGTA, 1 mM GTP and 10.2% glycerol. 100 µl of the diluted tubulin added with DAPI was immediately loaded into each of 6 wells of prewarmed (37°C) 96-well plate that has been pre-added with two wells of 10 µl PBS as negative control, two wells of 10 µl of paclitaxel as positive control (final concentration 3 µM) and two wells of 10 µl of recombinant human gamma-synuclein (final concentration 25 µM). The plate was immediately subjected for kinetic reading at 37°C by excitation at 360 nm and emission at 420 nm for 1hr. Polymerized microtubules in the

presence of gamma-synuclein were centrifuged for 90 min at $22,500 \times g$. Supernatants and pellets were separated and subjected to SDS-PAGE analysis. For scanning electron microscopy, microtubules from tubulin polymerization assay were centrifuged at $22,500 \times g$ for 20 min. The pellet was fixed with 2% glutaraldehyde and 1% tannic acid and subjected for microscopy analysis.

Antibody crosslinking assay

To prepare preformed paclitaxel stabilized microtubules, 8 μg of unlabeled tubulin with trace amount of Rhodamine-labeled tubulin (mass ratio = 120:1) was mixed with 5 μl of General Tubulin Buffer (80 nM Piperazine-N,N'-bis [2-ethanesulfonic acid] sequeisodium salt; 2.0 mM MgCl_2 ; 0.5 mM Ethylene glycol-bis (b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, pH 6.9) and 2 μl of cushion buffer (80 nM Piperazine-N,N'-bis [2-ethanesulfonic acid] sequeisodium salt; 2.0 mM MgCl_2 ; 0.5 mM Ethylene glycol-bis (b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, 60% v/v glycerol, pH 6.9]. Both General Tubulin Buffer and cushion buffer were purchased from Cytoskeleton, Inc. The mixture was incubated for 5 min at 37°C and immediately added with 100 μl of paclitaxel at 200 nM. The integrity of the preformed microtubules was checked using fluorescence microscope. To perform antibody crosslinking assay, 50 μl of preformed microtubules was mixed with 200 ng of either anti-tubulin or 200 ng anti-BSA antibodies or with 200 ng or 600 ng anti-gamma-synuclein antibodies. The bundle formation was examined 5 min later under fluorescence microscope (200 $\times$).

Microtubule bundling by gamma-synuclein and MAP2

10 μg tubulin was mixed with 4 μl the General Tubulin Buffer, 2 μl cushion buffer and (1) 2 μl or 4 μl of recombinant human gamma-synuclein (1 $\mu\text{g}/\mu\text{l}$), (2) 2 μl of recombinant MAP2 or BSA (1 $\mu\text{g}/\mu\text{l}$), (3) 2 μl of gamma-synuclein (1 $\mu\text{g}/\mu\text{l}$) plus 2 μl MAP2 (1 $\mu\text{g}/\mu\text{l}$), (4) 4 μl of gamma-synuclein (1 $\mu\text{g}/\mu\text{l}$) plus 2 μl MAP2 (1 $\mu\text{g}/\mu\text{l}$), or (5) 2 μl of BSA (1 $\mu\text{g}/\mu\text{l}$) plus 2 μl MAP2 (1 $\mu\text{g}/\mu\text{l}$). After the mixture was incubated at room temperature for 15 min, the sample was examined by fluorescence microscopy (200 $\times$).

Chamber perfusion assay

This assay was modified from kinesin motility assay protocol used for Kinesin Motility Assay Biochem Kit (Cytoskeleton Inc). All the working solutions including General Tubulin Buffer, blocking buffer and wash buffer was provided by the kit. Briefly, 4 μg of BSA (negative control), recombinant human gamma-synuclein, or recombinant human kinesin (positive control) were dissolved in 11 μl of General Tubulin Buffer. To coat the chamber, solutions were perfused into 3 individual perfusion chambers and incubated at room temperature for 5 minutes. After coating, 11 μl of blocking solution was perfused through perfusion chamber for 5 min to prevent non-specific binding. To test the binding activity, 10 μl of preformed fluorescent microtubules were perfused through perfusion chamber and incubated for 5 min, followed by two times of washing with 20 μl of wash buffer to remove unbounded microtubules. The chambers were then subjected to examination under fluorescence microscope (200 $\times$).

Cytoskeleton isolation

Cytoskeleton was isolated using Microtubules/Tubulin *In Vitro* Assay Kit (Cytoskeleton Inc.). Briefly, T47D cells grown on 10 cm cell culture plate were covered with warmed (37°C) Lysis and Microtubule Stabilization Buffer (component of the kit) along with ATP, GTP and phosphatase inhibitors, collected, homogenized with 25 G syringe and incubated for 10 min at 37°C . 95% cell rupture was achieved based on microscopy examination. The

homogenate was centrifuged at $50,000 \times g$ for 1 hr at 37°C. The supernatant and the pellet were subjected to western blot analysis.

***In vivo* microtubule bundle measurement**

GFP-tubulin HeLa cells were transfected with gamma-synuclein or empty vector as control. Three days later both cultures were treated with paclitaxel (10 μ M) for 5 hr to induce microtubule bundle formation. 10 fields were randomly chosen and subjected for fluorescence microscopy imaging (600 $\times$). All the images were taken using the same exposure time. The experiments were repeated twice. The intensity and the area of the microtubule bundle were measured using ImageJ software. The results were plotted as the percentage of microtubule bundle reduction mediated by gamma-synuclein overexpression versus vector controls. The P value was calculated using a two-tailed t test.

Results

Gamma-synuclein promoted tubulin polymerization *in vitro*

To test if gamma-synuclein can affect tubulin polymerization, we performed *in vitro* tubulin polymerization assays in the presence or absence of recombinant human gamma-synuclein protein. Paclitaxel was included as positive control. As shown in Figure 1A, the presence of recombinant gamma-synuclein protein significantly accelerated microtubule assembly. While level of such acceleration is not as high as that seen in paclitaxel treated samples, tubulin polymerization in both paclitaxel and gamma-synuclein containing samples reached maximum readout at nearly the same time point. To determine if microtubules formed in the presence of gamma-synuclein maintain normal ultrastructure, we performed scanning electronic microscopy on all samples. As might be expected, paclitaxel induced the formation of higher numbers of microtubules (Figure 1B-c) than gamma-synuclein (Figure 1B-b). When examined at higher magnification, the protofilaments of microtubules formed in the presence of gamma-synuclein appeared to be normal (Figure 1C-b).

Gamma-synucleins bound to microtubules *in vitro*

To examine if synucleins can directly bind to microtubules, we isolated polymerized tubulins from gamma-synuclein containing samples. Direct physical association was demonstrated by western blotting showing the presence of gamma-synuclein in microtubule-containing pellets (Figure 2A). To confirm this observation, we performed antibody-crosslinking assays. Since antibodies can crosslink targets, we tested if anti-gamma-synuclein antibodies can crosslink microtubules formed in the presence of gamma-synuclein. As further evidence, the microtubules in gamma-synuclein added samples were gathered into bundles upon anti-gamma-synuclein (Figure 2Bc-d) and anti-tubulin (Figure 2B-a, as positive control) but not anti-BSA (Figure 2B-b, as negative control) antibody treatment. The bundle formation became more apparent upon the increase in the amount of added anti-gamma-synuclein antibody. This result suggested that the gamma-synuclein is localized on the surface of microtubules and promotes their assembly through direct binding. To further prove this conclusion, we performed chamber perfusion assay in which preformed microtubules were forced to flow through a perfusion chamber coated with BSA or human recombinant gamma-synuclein or kinesin. Kinesin is a motor protein known to bind microtubules. It served as positive control here and BSA was used as a negative control. As shown in Figure 2C, while the microtubules did not bind to gamma-synuclein as strongly as they did to kinesin, the presence of microtubules in gamma-synuclein but not BSA coated chambers further indicated a direct physical association.

Gamma-synuclein altered the phenotype of microtubules formed in the presence of microtubule associated protein 2 (MAP2) *in vitro*

MAP2 is a neuronal microtubule regulatory protein that confers its microtubule-stabilizing effect via direct binding. As other microtubule associated proteins, it induces formation of microtubule bundles *in vitro* and *in vivo* [16-21]. Since gamma-synuclein was also found to bind and regulate microtubule assembly, we next examined if it could also induce microtubule bundling *in vitro*. As shown in Figure 3A, the additional of recombinant human gamma-synuclein protein to preformed microtubules induced microtubule bundling in a dose dependent manner. Furthermore, gamma-synuclein addition altered the phenotype of microtubules formed in the presence of MAP2 alone. As shown in Figure 3B-a, under tested conditions, MAP2 alone induced aggregations of short microtubule bundles, which were not affected by BSA addition (Figure 3B-b). However, simultaneous addition of gamma-synuclein and MAP2 led to the formation of long and thin microtubules bundles which resembles those formed in the presence gamma-synuclein alone (Figure 3B-c). With the increase in the gamma-synuclein concentration, the number of individual microtubules decreased but the bundles became thicker (Figure 3B-d). Since dimerization of microtubule associated proteins is a common mechanism for crosslinking microtubules, we examined the biophysical status of recombinant gamma-synuclein as well as endogenous gamma-synuclein isolated from cell lysate or mouse brain tissue. Using samples prepared under reducing conditions and boiling temperature for 10 min, we found that gamma-synuclein exists as monomers and dimers at different levels in both cell lysate and mouse brain samples (Figure 3C). In recombinant human gamma-synuclein sample, trimers (based on molecular weight) can also be found.

Gamma-synuclein bound to cytoskeleton and colocalized with microtubules *in vivo*

To test if synucleins could bind to microtubules in cells, we isolated actin/microtubule portion of cytoskeleton from T47D cells. T47D cells were examined here because it contains high level of endogenous gamma-synuclein (Figure 4A-a). AKT was probed as cytosolic protein control. Interestingly, oligomers of gamma-synuclein were detected only in the assay pellet which represents cell cytoskeleton but not in the supernatant which primarily represents the cytosolic compartment of cells (Figure 4A-b). Similar observations have been made using GFP-tubulin HeLa cells transiently transfected with gamma-synuclein (Supplemental Figure 1). Interestingly, exogenous expression of gamma synuclein in GFP-tubulin HeLa cells resulted in dimer formation rather than oligomers as seen in T47D cells. To further confirm direct interaction between gamma-synuclein and microtubule *in vivo*, we assessed the colocalization of gamma-synuclein with microtubule by confocal immunofluorescence staining. Because sample processing could easily damage microtubule structures and present difficulties for immunostaining, we used GFP-tubulin HeLa cells, which stably expressed GFP-tagged tubulin. Gamma-synuclein cDNA expression constructs or empty vector was transiently transfected into GFP-tubulin HeLa cells. Confocal microscopy examination showed that although gamma-synuclein displayed an overall cytosolic staining with no distinct microtubule distribution patterns, colocalization of gamma-synuclein and microtubule bundles could be identified in certain sublocation of both mono- and multi-nucleated HeLa cells, especially at perinuclear areas (Figure 4B).

The counteractive effect of gamma-synuclein on paclitaxel-induced microtubule abnormality *in vivo*

In our previous studies, we found that gamma-synuclein expression can enhance resistance to paclitaxel induced cell death in A2780 ovarian cancer cells. Supported by additional evidence mentioned above, we hypothesized that at least partial *in vivo* anti-paclitaxel effect was mediated through direct microtubule modification. To test this hypothesis, we examined if overexpressing gamma-synuclein can lead to microtubule changes in GFP-tubulin HeLa

cells upon paclitaxel treatment. As shown in Figure 5A, at 5 hr post paclitaxel treatment, microtubules formed thick bundles around nucleus in GFP-tubulin HeLa cells transiently transfected either with empty vector or vector containing human gamma-synuclein gene. Interestingly, the curve of the bundles surrounding nuclei were smoother in gamma-synuclein transfected cells than control cultures (Figure 5A-b vs. 5A-a). Since gamma-synuclein colocalized with microtubule bundles around nuclear area in GFP-tubulin HeLa cells (Figure 4), the reduced microtubule rigidity seen in gamma-synuclein expressing cells could be due to competitive binding of gamma-synuclein to microtubule bundles against paclitaxel. However, the indirect effect of gamma synuclein on microtubule rigidity cannot be ruled out due to the lack of evidence that higher level of gamma synuclein expression correlates with higher level of microtubule flexibility in individual transfected HeLa cells. In addition, we measured the intensity and the area of microtubule bundles in paclitaxel-treated vector controls cells or gamma-synuclein transfected cells. Between 20~40% of bundle reduction was observed upon exogenous expression of gamma-synuclein (Supplemental Figure 2). In another study, stable expression of gamma-synuclein significantly improved cell attachment upon paclitaxel treatment in A2780 cells (Figure 5B). In this experiment, trace amount of Alexa488 conjugated paclitaxel was mixed with unlabeled paclitaxel to visualize distribution of paclitaxel and paclitaxel-decorated microtubules. In contrast to control cells (A2780 parental), we found that A2780 cells stably overexpressing gamma-synuclein (A2780 gamma-syn cells) could still have full attachment (indicated by flattened cell morphology) and display relatively intact microtubule structure.

Gamma-synuclein overexpression may alter microtubule-dependent mitochondria trafficking *in vivo*

To further determine the physiological relevance of gamma-synuclein in microtubule regulation *in vivo*, we stained mitochondria in A2780 parental as well A2780 gamma-syn cells. Mitochondria are known to be anchored on and transported along microtubules. It is commonly used to understand microtubule mediated organelle trafficking. In parental A2780 cells, mitochondria distributed throughout cytosol (Figure 6 A1-A2). To our surprise, mitochondria in A2780 gamma-syn cells formed aggregates in perinuclear area (Figure 6 B1-B2). Microtubule-disabling agent nocodazole treatment completely dispersed these mitochondria aggregates (Figure 6C), indicating that the accumulation of mitochondria nearby nucleus was not caused by direct mitochondria fusion or aggregation. Instead, it is most likely due to microtubule mediated mitochondria clustering. To further prove this interpretation, we treated A2780 gamma-syn cells with paclitaxel at 10 nM. It is known that paclitaxel at this low concentration alters microtubule dynamics and related cellular events without elicit toxic effect. We found that similar to nocodazole, low dose paclitaxel treatment significantly reversed the mitochondria clustering in large population of A2780 gamma-syn cells (Figure 6D), confirming that mitochondria clustering induced by stable expression of gamma-synuclein indeed was due the changes occurred at microtubule levels.

Discussion

Of the three synuclein family members, alpha-synuclein is by far the most studied and the only one until this study that demonstrated a direct linked to microtubule regulations. By affinity column chromatography, recombinant and human brain alpha-synuclein were found to specifically bind to tubulins from mouse forebrain cytosolic extract [22, 23]. Direct association was further confirmed by co-immunoprecipitation analysis using animal brains tissue, coimmunofluorescent staining using neuronal cells as well as electronic microscopy examination on tubulins polymerized in the presence of alpha-synuclein [22, 23]. As a component of amyloid deposition in Alzheimer and Lewy bodies in Parkinson disease, aggregated alpha-synuclein coexists in the same complexes with microtubule associated

protein tau [24]. Alpha-synuclein seeds the fibrillization of tau *in vitro* and incubation of alpha-synuclein and tau promoted the fibrillization of both proteins [24].

Gamma-synuclein and cancer cell resistance to anti-microtubule agents

Until this study, little was known about the relationship between gamma-synuclein and microtubule. However, we and others have found that gamma-synuclein expression in cancer cells led to differential drug resistance [14, 15]. Using breast and ovarian cancer cell model we demonstrated that overexpressing gamma-synuclein rescued cancer cells from apoptotic death induced by anti-microtubule agents such as paclitaxel or vinblastine but not by that induced by DNA damage drugs such as etoposide and doxorubicin [14, 15]; findings that were later confirmed by Zhou and colleagues [14]. In their studies using breast cancer cell lines, the level of gamma-synuclein was highly correlated with paclitaxel- but not doxorubicin-induced cytotoxicity. Such drug resistance profile does not seem to match what was found by multiple drug resistance research, which is paclitaxel resistant cancer cell lines with enhanced drug efflux system are normally cross-resistant to doxorubicin and vinblastine [25]. Therefore, we hypothesized that gamma-synuclein may exert its anti-paclitaxel or anti-vinblastine effect through directly modifying microtubules.

In vitro evidence for the role of gamma-synuclein in microtubule regulation

Alpha-synuclein promotes tubulin polymerization *in vitro* [26] and C-terminal domain seems to be implicated for its effect. While the C-terminal portion of gamma-synuclein is much more distinct to alpha synuclein than its N-terminal part, we found that recombinant human gamma-synuclein can also accelerate tubulin polymerization and promote normal arrangement of protofilaments. Interestingly, as indicated by kinetic curve, both gamma-synuclein and paclitaxel added samples had similar acceleration rate, indicating the potential high efficacy of gamma-synuclein on seeding or stabilizing tubulin polymers. Since three types of *in vitro* binding assays showed that gamma-synuclein could directly associate with polymerized tubulins, the polymerizing effect of gamma-synuclein is mostly likely mediated through direct binding.

Another key finding from our *in vitro* studies is that gamma-synuclein can induce microtubule bundling. Bundling microtubules is a property possessed by typical microtubules associated proteins. It is believed that microtubule bundles are formed by dimerization of microtubule associated proteins which can crosslink individual microtubules [27]. Significant amount of dimers was found in recombinant human gamma-synuclein proteins used for our *in vitro* assays. Because all synuclein family members are highly heat resistant, gamma-synuclein dimers can often be detected using tissue and sometimes cell lysates via immunoblotting under reducing conditions. Its capability to dimerize and oligomerize was also reported by another group [28].

MAP2 is a well-studied neuronal microtubule associate protein that regulates neuron development through modifying microtubule networks. It binds to microtubules, promotes their assembly, stabilize and crosslink microtubules both *in vitro* and *in vivo*. Under our tested conditions, we found gamma-synuclein can alter the phenotype or microtubules bundles induced by MAP2. Unlike bundles formed in the presence of same mass concentration of gamma-synuclein, MAP2 induced short microtubule bundles that aggregated together. Gamma-synuclein addition prevented aggregation and induced formation of longer MT bundles. The molecular weight for MAP2 is predicted to be 280 kDa and for gamma-synuclein is 16 kDa. Under same mass concentration, the molar ratio between MAP2 and gamma-synuclein is 1:17.5. This suggested there were approximately 17-fold more of gamma-synuclein molecules than MAP2 molecules in assay conditions. If competitive binding to tubulin occurred between two proteins, excessive gamma-synuclein

molecules may be able block access of MAP2 and shift phenotype of microtubule bundles. However, this is a hypothetical interpretation.

***In vivo* evidence for the role of gamma-synuclein in microtubule regulation**

Although *in vitro* assays suggested that gamma-synuclein can affect the properties of microtubule, overexpressing gamma-synuclein *in vivo* did not seem to dramatically alter the microtubule structure. No apparent microtubule bundling was seen in GFP-tubulin HeLa cells transfected with gamma-synuclein (data not shown). In addition, immunostaining found that gamma-synuclein distributed throughout cytosol and did not show pattern of microtubule network. However, when overexpressed in GFP-tubulin HeLa cells, gamma-synuclein was found to colocalize with the microtubule dense around perinuclear area. When treated with paclitaxel, the thick bundles formed in this area appeared to be more flexible than those formed in control cells. We also demonstrated that ovarian cancer cells, which stably overexpressing gamma-synuclein, displayed significantly improved microtubule structure than control cells upon paclitaxel treatment. These observations suggest that overexpression of gamma-synuclein may reduce cell chemo-sensitivity through decreasing microtubule rigidity.

Microtubule is the major structural network for intracellular organelle trafficking, including mitochondria transportation. Mitochondria perinuclear aggregation can occur either by unbalanced anterograde/retrograde transport [29] or unbalanced fusion/fission machinery [30]. In A2780 cells stably transfected with gamma-synuclein, we observed severe mitochondria clustering around nucleus. The fact that both nocodazole and paclitaxel treatment dispersed mitochondria clusters proved that this is a microtubule associated phenomenon, but not due to mitochondria fusion. Electron microscopy analysis of mitochondria in A2780 gamma-syn cells also supported this conclusion. Mitochondrial enlargement (sign of mitochondria fusion) was not found in A2780 gamma-syn cells except for their condensed gathering, indicating stable expression of gamma-synuclein *in vivo* may have modified mitochondria trafficking on microtubules. Actually our observation is similar to another finding in which overstabilizing microtubules by overexpressing other microtubule associated proteins decreased anterograde transport and caused mitochondria accumulate at the cell center [31].

In conclusion, we believe gamma-synuclein is a functional microtubule regulating protein that share similar properties with other microtubule associated proteins. However, it does not contain microtubule binding domain shared by MAP2 or tau [32]. Unlike typical microtubule associated proteins, gamma-synuclein is known to have other functions such as binding to heat shock protein and acting as chaperone component [33]. How this small molecule coordinates and switches from one role to other will need to be further elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Gamma-synuclein promoted microtubule polymerization *in vitro*

A) *In vitro* tubulin polymerization assay shows that 25 μ M recombinant gamma-synuclein protein significantly accelerated tubulin polymerization ($\blacktriangle$) compared to PBS control ($\bullet$). 3 μ M paclitaxel was used as positive control ($\blacksquare$). B) Scanning electron microscope pictures (4,000 $\times$) of polymerized microtubules formed in the presence of PBS (a), gamma-synuclein (b) and paclitaxel (c). C) Higher magnification view (80,000 $\times$) microtubules formed in the presence of PBS (a), gamma-synuclein (b) and paclitaxel (c).

Figure 2. Gamma-synuclein bound to microtubule *in vitro*

A) Microtubules from tubulin polymerization assay were pelleted and subjected to western blot analysis. Tubulin polymerization in the presence of alpha-synuclein was also performed and microtubules was pelleted and loaded in the same gel to act as negative control for the specificity of anti-gamma-synuclein antibody. Both tubulins and gamma-synuclein can be detected in the pellet, indicating the direct binding between tubulin and gamma-synuclein. B) Anti-gamma-synuclein antibody can cross link microtubules formed in the presence of gamma-synuclein. All the microtubules shown in a, b, c and d were formed in the presence of gamma-synuclein. As expected, anti-tubulin (0.2 μ g) crosslinked the microtubules into bundles (panel (a) shows the positive control) but anti-BSA antibody (0.2 μ g) does not (panel (b) represents the negative control). Panels c and d (0.2 μ g and 0.4 μ g anti-gamma-synuclein antibody was added, respectively) show crosslinked microtubules in a dose dependent manner, indicating the presence of gamma-synuclein on the surface of microtubules. C) Demonstrates that microtubules can bind to immobilized gamma-synuclein. Panel (a) chambers coated with BSA to serve as a negative control; (b) chambers coated with gamma-synuclein; and (c) chambers coated with kinesin to serve as positive control.

Figure 3. Gamma-synuclein induced microtubule bundle formation and altered the phenotype of the microtubules formed in the presence of MAP2

A) Microtubules form bundles in the presence of gamma-synuclein (c: 15.6 μ M & d: 25 μ M) but not BSA (b). Panel (a) is a control sample in which no protein was added. B) Tubulin forms aggregations in the presence of MAP2. Adding BSA did not prevent tubulin aggregation in the presence of MAP2 (b). However, gamma-synuclein addition (c: 12.5 μ M & d: 20.8 μ M) prevented aggregates formation and promoted the polymerization of tubulin into longer microtubules (c) and bundles (d). C) Gamma-synuclein was mainly found as monomers in A2780 cell lysate, dimers in mouse brain tissue lysate and monomers/dimers/trimers in human recombinant gamma-synuclein. (All the samples are processed in reducing buffer with 100 mM DTT and heated at 100°C for 10 min).

Figure 4. Gamma-synuclein bound to cytoskeleton and colocalized with microtubule *in vivo*

A) Gamma-synuclein binds to cytoskeleton *in vivo*. (a) Endogenous gamma-synuclein expression is shown for three cancer cell lines. (b) Cytoskeleton isolated from T47D cells were subjected to western blot analysis. AKT was probed as cytosolic protein control. Oligomers of gamma-synuclein were detected only in assay pellet which represents cell cytoskeleton but not in the supernatant which primarily represents the cytosolic compartment of cells. B) Gamma-synuclein and tubulin were found to colocalize in GFP-tubulin HeLa cells transfected with a gamma-synuclein construct (images are at 400 $\times$). GFP-tubulin HeLa cells transfected with empty vector was included as negative control (a, b, c). Panels a, d, g: GFP tubulin; Panels b, e, h: gamma-synuclein; Panels e, f, i: merged image. L: left cell (Pearson's correlation = 0.27), R: right cell (Pearson's correlation = 0.74). Arrows highlight examples of apparent co-localization of gamma-synuclein and microtubules.

Figure 5. Gamma-synuclein altered microtubule properties *in vivo*

A) Paclitaxel treatment (10uM, treated for 5hr) results in different morphology in microtubule bundles formed in empty vector-transfected (a) and gamma-synuclein transfected (b) GFP-tubulin HeLa cells. *Green: microtubules; Blue: DAPI. B) Gamma-synuclein stably transfected A2780 cells (b) are more resistant to paclitaxel treatment (1uM, treated for 16hr) and display more integrated microtubule structure than parental A2780 cells (a). In this experiment, trace amount of Alexa488 conjugated paclitaxel (indicated by green color) was mixed with unlabeled paclitaxel to visualize distribution of paclitaxel and paclitaxel-decorated microtubules. * The expression level of gamma-synuclein expression in each transfected cell lines was shown by western blot analysis on the left.

Figure 6. Stable expression of gamma-synuclein in A2780 cells led to mitochondria clustering
 Compared to parental A2780 cells (A-1), mitochondria in gamma-synuclein transfected cells clustered at perinuclear area (B-1). These clusters were disrupted upon nocodazole (1 μ M, treated for 1.5 hr) (C) or low concentration paclitaxel treatment (10 nM, treated for 1.5 hr) (D). Electron microscopy images of mitochondria (13,000 $\times$) in parental A2780 cells and A2780 gamma-syn cells were shown in panel A-2 and B-2, respectively.