γ-Synuclein Promotes Cancer Cell Survival and Inhibits Stress- and Chemotherapy Drug-induced Apoptosis by Modulating MAPK Pathways

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Synucleins are a family of highly conserved small proteins predominantly expressed in neurons. Recently we and others have found that γ-synuclein is dramatically up-regulated in the vast majority of late-stage breast and ovarian cancers and that γ-synuclein over-expression can enhance tumorigenicity. In the current study, we have found that γ-synuclein is associated with two major mitogen-activated kinases (MAPKs), i.e. extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1), and have shown that over-expression of γ-synuclein leads to constitutive activation of ERK1/2 and down-regulation of JNK1 in response to a host of environmental stress signals, including UV, arsenate, and heat shock. We also tested the effects of γ-synuclein on apoptosis and activation of JNK and ERK in response to several chemotherapy drugs. We have found that γ-synuclein-expressing cells are significantly more resistant to the chemotherapeutic drugs paclitaxel and vinblastine as compared with the parental cells. The resistance to paclitaxel can be partially obliterated when ERK activity is inhibited using a MEK1/2 inhibitor. Activation of JNK and its downstream caspase-3 by paclitaxel or vinblastine is significantly down-regulated in γ-synuclein-expressing cells, indicating that the paclitaxel- or vinblastine-activated apoptosis pathway is blocked by γ-synuclein. In contrast to paclitaxel and vinblastine, etoposide does not activate JNK, and γ-synuclein over-expression has no apparent effect on this drug-induced apoptosis. Taken together, our data indicate that oncogenic activation of γ-synuclein contributes to the development of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain chemotherapeutic drugs.

Breast carcinoma is the second leading cause of cancer-related deaths in women of the Western world. In the United States alone over 180,000 new cases are diagnosed annually and more than 40,000 women die from this disease each year (1). Epithelial ovarian cancer continues to be the leading cause of death from gynecologic malignancies in the United States (1, 2). One woman in 70 in the U.S. will develop ovarian cancer in her lifetime, and one woman in 100 will die of this disease. Breast and ovarian cancer etiology are multifactorial, involving environmental factors, hormones, genetic susceptibility, and genetic changes during progression. Both cancers are a heterogeneous group of tumors with no unifying molecular alteration yet identified. A certain number of breast and ovarian cancer cases (~5–10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as BRCA1 and BRCA2 (reviewed in Ref. 3). However, the majority of the tumors occur in women with little or no family history, and the molecular basis of these sporadic cancers is still poorly defined.

In an effort to identify other genes involved in the development and/or progression of breast and ovarian cancer, we and others used differential gene expression approaches and have found that γ-synuclein, initially termed breast cancer-specific gene 1 (BCSG1),§ is up-regulated in the majority of late-stage breast (4, 5) and ovarian cancer (4).¶ In addition, we showed that there was a correlation between γ-synuclein expression in breast ductal carcinomas and the staging of the cancer suggesting that γ-synuclein may be a potential marker for both late stage breast and ovarian cancer. Additional studies have revealed that γ-synuclein over-expression leads to increased invasiveness of breast tumor cells (6) and stimulated cell proliferation (7). Synucleins are a family of small, highly soluble proteins that are predominantly expressed in neurons. The functions of the synucleins are not entirely understood. There are four known members: α-synuclein (also referred to as synellin or non-Δβ component of Alzheimer’s disease (AD) amyloid precursor protein) and β-synuclein (also referred to as phosphoneuroprotein 14) are neuronal proteins primarily expressed in brain and are

The abbreviations used are: BCSG1, breast cancer-specific gene 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-JUN N-terminal kinase; MEK, MAPK kinase; LBs, Lewy bodies; AD, Alzheimer’s disease; pNA, p-nitroaniline; PD, Parkinson’s disease; PRC, protein kinase C; PARP, poly(ADP-ribose) polymerase; CMV, cytomegalovirus; PBS, fetal bovine serum; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase; GST, glutathione S-transferase.


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predominantly found at axonal terminals. γ-Synuclein (also known as persyn) is predominantly expressed in certain regions of the peripheral nervous system such as dorsal root ganglia and trigeminal ganglia. Synoretin, the newest member of the synuclein family, is expressed at high levels in the retina and at lower levels in the brain (8, 9). The synuclein proteins contain several repeated domains that display variations of a KTKEGV consensus sequence. The β-synuclein protein contains five of these domains, whereas the α- and γ-synucleins have six. Interestingly, the third domain of each protein is completely conserved, and this same type of domain is present in proteins of the Rho family (10). Another type of organization of the synuclein proteins that has been noted is an 11-residue repeat. This motif, repeated six to seven times in the N-terminal portion of the protein, is reminiscent of the amphiathic α-helical domains of the apolipoproteins and suggests possible lipid binding properties (11).

The γ-synuclein gene maps to 10q23, is composed of five coding exons, and is transcribed into an mRNA of ~1 kb (8). The human γ-synuclein is 127 amino acids long and has 87.7% and 83.3% identity to the mouse and rat proteins, respectively. In addition, comparison of the amino acid sequences indicates that γ-synuclein is highly homologous to α-synuclein and β-synuclein except for the last 27 amino acids of γ-synuclein. Overall, γ-synuclein shares 54%, 56%, and 84% amino acid sequence identity with α-synuclein, β-synuclein, and synoretin, respectively (8, 9).

Among the synucleins, α-synuclein is the best characterized because of its significant role implicated in neurodegenerative diseases (12). Mutations in the α-synuclein gene have been identified in rare kindreds with Parkinson’s disease (PD) (12–14), and intracytoplasmic aggregates comprised of α-synuclein fibrils are characteristic of several neurodegenerative diseases as exemplified by the intraneuronal Lewy bodies (LBs), neuroaxonal spheroids, and dystrophic neurites (i.e., Lewy neurites) that are prominent in PD, LB variant of Alzheimer’s disease (AD), and dementia with LBs (14–19).

The normal physiological functions of synucleins are not well characterized. The N-terminal portion of α-synuclein (residues 1–61) shares 40% amino acid homology with members of the 14-3-3 protein family (20). The 14-3-3 family of proteins helps regulate many different signal transduction pathways and is thought to act by directly binding to various protein kinases and bringing them into close proximity with substrate and regulatory proteins. 14-3-3 proteins bind to phospho-serine residues critical for the functions of many kinases and phosphatases that are involved in diverse cell functions (21–24). Like the synucleins, 14-3-3 proteins are ubiquitously expressed in the brain and have been shown to associate in a chaperone-like manner with PKC, BAD, ERK, and RAF-1 (16, 21). α-Synuclein binds 14-3-3 as well as to PKC, BAD, ERK, and the microtubule-associated protein tau (25). In addition to shared regions of homology to 14-3-3, α-synuclein as well as β- and γ-synuclein also appear to act as a protein chaperone, at least in vitro, by disrupting protein aggregation (26).

To help further unravel the function of γ-synuclein and establish its role in the oncogenesis of breast and ovarian cancer, we searched for proteins that could interact with γ-synuclein and identified the MAPKs ERK1/2 and JNK1. In this study we provide evidence that γ-synuclein contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways. In addition, we observed that paclitaxel- or vinblastine-induced cell death is protected by γ-synuclein, indicating that chemotherapeutic drugs that take effect through activating the JNK apoptosis pathway may not be effective for cancer with high γ-synuclein expression.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Paclitaxel, vinblastine, and etoposide were purchased from Sigma (St. Louis, MO). The MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI). Anti-ERK1, anti-ERK2, and anti-JNK1 antibodies and normal IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2 and anti-PARP antibodies were obtained from Cell Signaling (Beverly, MA). The mouse antibody Syn303 was raised to recombiant human α-synuclein but recognizes α-, β-, and γ-synuclein. γ-2 is a rabbit polyclonal antibody raised to recombinant human γ-synuclein that specifically recognizes human γ-synuclein (27). Mouse monoclonal antibodies Syn204 and Syn207 were raised against human α- and β-synucleins, respectively.

**Cell Culture and Transfection—**Ovarian cancer cell lines A2780 and OVCAR5 were maintained in 10% FBS Dulbecco’s modified Eagle’s medium and 10% FBS RPMI 1640, respectively. HEK293, human embryonic kidney cells, were maintained in 10% FBS Dulbecco’s modified Eagle’s medium supplemented with sodium pyruvate and non-essential amino acids. To create the CMV plasmid for establishing stable cell lines over-expressing human α-, β-, or γ-synuclein, human cDNAs were amplified by PCR and subcloned into pCMV (Invitrogen). GenePorter Transfection Reagent (GTS Inc., San Diego, CA) was used for transfection, and stable cell lines were selected by G418 (Invitrogen). Expression of human α-, β-, or γ-synuclein was confirmed by immunoblotting with Syn204, Syn207, or γ-2 antibody, respectively.

**Cell Treatment with UV and Other Stress Signals or Chemotherapeutic Drugs—**For UV treatment, cells at 70–80% confluence were washed once with PBS before UV irradiation (254 nm, 20 J/m 2). Complete medium was added to the plates after treatment. The intensity of the UV light source was measured with a BLAK-RAY meter (UVP, Inc., San Gabriel, CA) prior to each experiment. Heat-shock was carried at 42°C for 15 min. 50 μl sodium arsenite was used to treat the cells for 6 h. Paclitaxel, vinblastine, etoposide, and U0126 were dissolved in Me2SO, and cells were treated with various concentrations of the drugs as indicated in each experiment.

**Co-immunoprecipitation—**Cells at 70–80% confluence were washed twice with ice-cold n-PBS before scraping on ice with lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM sodium pyrophosphate; 1 mM sodium β-glycerophosphate; 5 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 1 tablet of protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) per 40 ml of lysis buffer). Cellular debris was removed by centrifugation (14,000 × g for 15 min at 4°C) and precleared with protein G-agarose (Invitrogen, Rockville, MD). Protein concentrations were determined with Bio-Rad DC protein assay reagents. Syn303 (3 μl of ascites) or control IgG (3 μg) were preincubated in 500 μl of PBS with 50 μl of anti-ERK or anti-JNK antibodies and washed once with PBS before incubation with 300 μg of total cellular lysate for 4 h at 4°C. The beads were washed four times with the lysis buffer, resuspended in 50 μl of n-PBS sample buffer before boiling for 5 min. 10 μl of immunoprecipitates was separated by SDS-PAGE electrophoresis on 4–20% linear gradient Tris-HCl ready gels (Bio-Rad).

**Immunoblotting and Data Quantification—**Proteins separated on SDS-PAGE gels were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The primary antibodies were diluted 1:1000, and the horseradish peroxidase-conjugated secondary antibodies were diluted 1:10,000 (Amersham Biosciences, Piscataway, NJ). PerkinElmer Life Sciences Renaissance Enhanced Luminol Reagents (Boston, MA) were used as substrates for detection. For re-use of the same membrane with another primary antibody, Restore Western Blot Stripping buffer (Pierce, Rockford, IL) was used to strip the membrane. The results of immunoblotting were quantitated using the program IMAGE (National Institutes of Health) for the integrated density of each band.

**JNK Kinase Activity Assay—**The kinase activity of JNK was measured using the SAPK/JNK assay kit (Cell Signaling Technologies). Briefly, 250 μg of cell lysate (1 μg/μl protein) was incubated with 1 μg of fusion protein beads (20 μl) overnight at 4°C. After washing, the proteins on the beads were incubated in the kinase reaction buffer supplemented with 100 μM ATP for 30 min at 30°C. To measure the JNK activity, the phosphorylated c-JUN was detected by SDS-PAGE and immunoblotting with the specific antibody (Cell Signaling Technologies).

**Cell Viability Assay—**Cell viability was determined by Trypan blue exclusion assay and/or WST-1 assay. For Trypan blue assay, cells were...
stained with 0.2% Trypan blue for 2–5 min. The number of viable cells (non-stained) and dead cells (stained) were counted under a microscope using a cell hemacytometer. For the WST-1 assay, cells under different culture conditions were incubated with WST-1 (Roche Molecular Biochemicals) for 4 h. Cleavage of WST-1 to formazan was monitored at 450 nm using a microplate reader.

Caspase Activity Assay—Colorimetric CaspACE assay (Promega) was used to detect the caspase-3 activity. Briefly, pNA released from the substrate Ac-DEVD-pNA by caspase-3 in the cell lysate was monitored at 405 nm using a microplate reader.

Statistical Analysis—Where indicated, a two-tailed Student t test was used to test for significance.

RESULTS

Expression of Synuclein in Tumor Cell Lines—We have previously reported that γ-synuclein is highly expressed in the vast majority of late-stage breast and ovarian tumors (4), suggesting a potentially important role for γ-synuclein in the development of these diseases. To help unravel the function of γ-synuclein, we established several in vitro models. The ovarian tumor cell lines A2780 and OVCAR5, which express low levels of γ-synuclein, as well as kidney HEK293 cells, which do not express detectable levels of γ-synuclein, were transfected with CMV-γ-synuclein or with vector alone and were selected with G418. Resistant colonies were screened by Western blotting for stable expression of γ-synuclein protein, and positive colonies were pooled into A2780gam, OVCAR5gam, and 293gam cell lines (Fig. 1). Cell lines stably expressing γ- and α-synucleins were also derived from A2780 cells as described for the γ-synuclein expressing lines (Fig. 1). Like A2780gam, OVCAR5gam, and 293gam cells, there were no obvious alterations in cell doubling time of the A2780alpha or A2780beta cell lines (data not shown).

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ERK1/2 and JNK1 kinase but not with the p38 kinase (Fig. 2). We also confirmed that α-synuclein is associated with ERK1/2 as well with JNK1 (Fig. 2), which is consistent with the recent studies using neuro2a, a neuronal cell line (29). These data indicate that γ- and α-synucleins can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.

Elevated Activity of ERK in Cells Over-expressing γ-Synuclein—We next evaluated whether these protein inter-
actions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ-synuclein, the activated ERK1/2 was increased 2- to 3-fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (Fig. 3). In contrast, α- and β-synucleins appeared to have little or no effect on the activity of ERK1/2 (Fig. 3A), although α-synuclein was also found to be associated with ERK (as described above and shown in Fig. 2) in A2780 cells. In HEK293 cells, the basal level of ERK activation is undetectable and γ-synuclein over-expression does not increase its activation level (Fig. 3B). Structural analysis indicated that γ-synuclein does not contain any kinase domain, suggesting that the activation of ERK is mediated by other kinase. Because MEK1/2 is required for the activation of ERK1/2 in response to many mitogens, we determined whether MEK1/2 is still required for γ-synuclein-mediated activation of ERK1/2. When cells over-expressing γ-synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (Fig. 4A). We further studied the relation of γ-synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ-synuclein and ERK1/2 was still present (Fig. 4B). These data indicate that γ-synuclein

may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ-synuclein.

Down-regulation of JNK Activation by γ-Synuclein in Response to Different Stress Signals—JNK is activated by stress signals, including UV, which leads to mitochondria-mediated apoptosis (30). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ-synuclein was over-expressed or not (Fig. 5). JNK was highly activated in the parental cells when treated with UV (Fig. 5). In cells over-expressing γ-synuclein, the activation of JNK was almost completely blocked in A2780/gam cells (p < 0.05) and was down-regulated by ~50% in OVCAR5/gam cells when treated with UV (Fig. 5) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ-synuclein-specific, because over-expression of α- and β-synucleins appeared to have little or no effect on the activation of JNK by UV (Fig. 5 and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific or it could be the high endogenous γ-synuclein.
expression in OVCAR5 cells. Similarly, the activation of JNK by sodium arsenate was blocked to different extents by \( \gamma \)-synuclein in 293/gam, OVCAR5/gam, and A2780/gam cells (Fig. 6). Collectively, these data indicate that stress-induced activation of JNK can be blocked by \( \gamma \)-synuclein overexpression in a variety of cell lines.

\( \gamma \)-Synuclein May Protect Paclitaxel (Taxol)-induced Cell Death by Regulating JNK and ERK Activities—Based on the data presented above, we hypothesized that \( \gamma \)-synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated \( \gamma \)-synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, cell survival and cell death are regulated by the counterbalance between the

![Graph showing JNK activation](image)

The graph above the blots is the average ± S.E. of three independent experiments. * significant inhibition of JNK activation compared with that in the parental cells \((p < 0.05)\).
Taxol does not affect ERK activity or produced cell death and is partially mediated by ERK activation. By trypan blue staining (shown) and WST-1 assays (not shown). The or absence of the MEK1/2 inhibitor, U0126. Cell death was determined to inhibition of the mitochondria-associated caspase pathway may (Ref. 31 and data not shown) and PI3K-AKT (32, 33), the UV treatment also activates the cell survival pathways ERK and AKT in A2780 cells. In initiation of the mitochondria-associated caspase pathway may (Ref. 31 and data not shown) and PI3K-AKT (32, 33), the UV treatment also activates the cell survival pathways ERK and AKT in A2780 cells. In (10\textmu M) were treated with Taxol (30 \textmu M) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. B, cell death induced by Taxol was significantly reduced in cells over-expressing \(-\)-synuclein. A2780 and A2780/gam cells treated with Taxol for 48 h in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average \pm S.E. of three independent experiments. * significant difference in cell death between A2780 and A2780/gam cells (p < 0.05).

When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced by \(-25\%\) in A2780 cells but was nearly doubled in A2780/gam cells (Fig. 7B). These data suggest that enhanced cell survival in \(-\)-synuclein over-expressing cells is partially mediated by activation of ERK.

To determine whether the protective role of \(-\)-synuclein on cell survival is also mediated through down-regulating JNK-associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (34, 36), JNK was activated in A2780 cells when treated with 30 \mu M Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol (p < 0.05) (Fig. 8A). In the parental A2780 cells, caspase-3 was highly activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in \(-\)-synuclein over-expressing ovarian cancer cells (p < 0.05) (Fig. 8B). These data indicate that the Taxol-activated JNK-mediated caspase apoptotic pathway was significantly attenuated in cells over-expressing \(-\)-synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by \(-\)-synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

\(-\)-Synuclein Overexpression Leads to Protection from Vinblastine but Not Etoposide-induced Cell Death—To demonstrate whether the effects of \(-\)-synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents. Taxol binds to microtubule polymers, whereas vinblastine binds to monomers and dimers. When treated with vinblastine (0.1, 1.0, or 10 \mu M for 30 h), cell death in A2780/gam cells was significantly lower (p < 0.05 for all the three concentrations tested) as compared with the parental cells (Fig. 9A). Consistent with other studies using a variety of tumor cell lines (36–39), vinblastine strongly activated JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by \(-\)-synuclein over-expression (Fig. 9B). Furthermore, we observe that treatment with vinblastine results in a 2-fold increase in phosphorylated JNK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (Fig. 9B). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (Fig. 9, A and C). These data indicate that suppression of vinblastine-induced cell death by \(-\)-synuclein may be mediated by inhibition of JNK activation.

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (37, 40–42). When treated with 1, 10, or 100 \mu M etoposide for 56 h, there was no significant difference in cell survival between A2780 and A2780/gam cells (Fig. 10A). As might be predicted, JNK was not activated in response to etoposide treatment (Fig. 10B). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down-regulated within 30 min of treatment with 10 or 100 \mu M of etoposide (Fig. 10C). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and \(-\)-synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.
DISCUSSION

Synucleins are highly soluble proteins, and their biological and biochemical functions are not entirely understood. Previous studies have suggested that they may be involved in neuron development and function (43). The involvement of \( \gamma \)-synuclein in human neoplastic diseases came to light when \( \gamma \)-synuclein was isolated from a human breast tumor cDNA library and was shown to be over-expressed in infiltrating ductal carcinomas (4, 5) and ovarian cancer (4). Additional studies have suggested that \( \gamma \)-synuclein may be involved in enhancing cell motility and metastasis, in breast (6) and ovarian cancer cells as analyzed both in vitro and in nude mouse models in vivo. We have found that oncogenic activation of \( \gamma \)-synuclein is independent of gene mutations or gene amplification. Liu and colleagues have recently reported that hypomethylation of the \( \gamma \)-synuclein gene CpG island promotes its aberrant expression in breast (45) and ovarian carcinomas.\(^4\) In the present studies, we showed that \( \gamma \)-synuclein over-expression activates the survival factor ERK1/2 and blocks the activation of JNK. Activation of JNK can initiate the mitochondria-involved caspase apoptosis pathway (30). Therefore, we propose that \( \gamma \)-synuclein, in its oncogenic form (over-expressed) contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways (Fig. 11). In addition, we observed that Taxol- and vinblastine-induced cell death is protected by \( \gamma \)-synuclein indicating that anti-cancer drugs that take effect through activating the JNK/caspase apoptosis pathway may not be effective for cancers with high \( \gamma \)-synuclein protein levels.

The effects of \( \gamma \)-synuclein on ERK1/2 and JNK signaling in ovarian cancer cells appear to be specific for \( \gamma \)-synuclein, be-

\(^3\) W. Bruening and A. K. Godwin, unpublished data.

\(^4\) A. Gupta, A. K. Godwin, L. Vanderveer, A. P. Lu, and J. Liu, submitted for publication.
cause over-expression of the α- and β-synucleins in A2780 cells had little or no effect on these MAPKs (Figs. 3 and 5). Even though α- and β-synucleins are expressed in a significant fraction of ovarian tumors (4), we did not observe a discernable phenotype associated with over-expression in cultured cells. The reasons for these differences are not readily apparent. However, we have recently observed that the subcellular localization of γ-synuclein in tumor cells may be altered. Previous studies have shown that γ-synuclein is diffusely distributed in the cytoplasm of peripheral neurons, although it is also expressed in the brain (46). We have found in tumor cells, which over-express the wild-type γ-synuclein, that the protein tends to accumulate in the nuclei. 2 This may also help explain why only a portion of ERK1/2 and JNK co-immunoprecipitates with γ-synuclein (Fig. 2), because the cytoplasmic levels are low in A2780 cells. Furthermore, the amino acid sequences of α- and β-synucleins are more closely related to each other than γ-synuclein (8, 9), and the γ-synuclein protein is the least conserved of the synuclein proteins (8, 9). Therefore, it is possible that the conserved sequences of α, β, and γ-synuclein may be involved in the interaction with ERK1/2 and that the non-conserved regions (predominantly the C terminus) may contribute to activation of ERK in a cell type-specific manner. Therefore, the effects that we are observing may be both cell type- and isoform-specific. Additional studies will be needed to better resolve these differences.

Even though we have made some significant observations in this study, the biochemical function of γ-synuclein remains largely unknown. Many of our approaches have come from the study of α-synuclein. α-Synuclein, the most extensively studied synuclein, is the major component of Lewy bodies in sporadic PD, dementia with LBs, and a subtype of Alzheimer’s disease known as the LB variant of Alzheimer’s disease (19, 47, 48). Mutations in the α-synuclein gene have also been linked to familial Parkinson’s disease (49–51). Based on homology to 14-3-3 protein and in vitro assay, synucleins are hypothesized to be proteins with chaperone properties (20, 26). α-Synuclein has been shown to associate with many of the proteins that interact with 14-3-3 proteins, including PKC, BAD, and ERK (16, 21, 25). α-Synuclein has also been shown to associate with ERK2 (28) and may reduce the phosphorylation of MAPKs in neurons (29), whereas synoretin can activate the Elk1 pathway.
when transfected in HEK293 cells (9). We have demonstrated in ovarian cancer cells that both \( \gamma \)-synuclein, A2780 and A2780/gam cells treated with etoposide (1, 10, and 100 \( \mu \)M) for 56 h in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. B, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 \( \mu \)M, and 100 \( \mu \)M) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. C, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 and 100 \( \mu \)M) in the absence or presence of U0126 (10 \( \mu \)M) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

**Fig. 10.** Effects of \( \gamma \)-synuclein overexpression on etoposide-induced cell death and activation of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express \( \gamma \)-synuclein. A2780 and A2780/gam cells treated with etoposide (1, 10, and 100 \( \mu \)M) for 56 h in the presence or absence of MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. B, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 \( \mu \)M, and 100 \( \mu \)M) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. C, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 and 100 \( \mu \)M) in the absence or presence of U0126 (10 \( \mu \)M) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

**Fig. 11.** A proposed model illustrating how \( \gamma \)-synuclein promotes cell survival by modulating the ERK and JNK pathways.

When transfected in HEK293 cells (9). We have demonstrated in ovarian cancer cells that both \( \gamma \)- and \( \alpha \)-synucleins can associate with ERK1/2 (Fig. 2). However, only \( \gamma \)-synuclein but not \( \alpha \)-synuclein lead to constitutive activation of ERK indicating that the function of \( \alpha \)-synuclein might be different in cancer cells versus neuronal cells. Studies are underway to determine whether this constitutive activation is dependent on the C-terminal sequences, which are unique to \( \gamma \)-synuclein.

The second prominent observation we made in this study was that ectopic expression of \( \gamma \)-synuclein resulted in enhanced resistance to the chemotherapeutic drugs Taxol and vinblastine (Figs. 7–9). In contrast, when these same cells were treated with etoposide, a DNA-damaging agent, \( \gamma \)-synuclein over-expression did not enhance cell survival (Fig. 10). There are two main apoptosis pathways initiated from the cell surface membrane, one is initiated by Fas and other death receptors, while the other is initiated by stress signals (30). JNK activation is an essential component of the latter apoptotic signaling (30, 52). In addition, many chemotherapy drugs also take effect partly through activating the JNK signaling (34–36). Our data indicate that abnormal over-expression of \( \gamma \)-synuclein might be one such mechanism in breast and ovarian cancer that permits tumor cells to overcome the JNK-activated apoptotic signaling (30). Both Taxol and vinblastine robustly induced JNK activity in A2780 cells, whereas the etoposide treatment induced such activity very little (Figs. 8–10). In \( \gamma \)-synuclein over-expressing cells, this induction by Taxol and vinblastine was suppressed 2- to 3-fold and cell survival was dramatically enhanced. Taken

**Fig. 11.** A proposed model illustrating how \( \gamma \)-synuclein promotes cell survival by modulating the ERK and JNK pathways.
together, these results indicate that the cell death in ovarian cancer cells induced by Taxol, vinblastine, or other drugs that induce JNK may be protected by γ-synuclein. Furthermore, it is interesting to note that only ~35% of breast tumors and ~50% of all epithelial ovarian tumors possess mutations in the TP53 gene (53, 54). Because JNK activation in UV- or Taxol-treated cells is independent of DNA damage (31, 55–57), it is interesting to speculate that over-expression of γ-synuclein may promote tumor cell survival in the presence of p53 wild-type cells. Because γ-synuclein is not expressed in normal breast and ovarian epithelial cells but is expressed in the majority of late-stage breast and ovarian cancers (4, 5), it may be a very promising target to develop drugs for the therapies of these diseases.

Our results of γ-synuclein in cancer cells seem different from those of α-synuclein in neuronal cells. It remains to be determined whether these differences are caused by the different cellular context between cancer and neuronal cells, by different subcellular localization of the proteins in various normal and tumor cell types, or by the innate difference in protein functions between γ- and α-synucleins. α-Synuclein overexpression in HEK293 cells or A2780 cells does not induce apoptosis (29). In neurons, mutant α-synuclein may accelerate apoptosis (58–61) while wild-type α-synuclein may induce or block apoptosis, depending on the path of apoptosis induction (29, 58–60, 62). In Parkinson’s diseases, mitochondrial dysfunction and oxidative stress are believed to be the two main causes for neuronal death (63). The apoptotic role of wild-type α-synuclein in neuronal apoptosis may be secondary by disrupting mitochondria and causing oxidative stress (64). There is evidence that the roles of synuclein in neurodegenerative diseases appear to be quite different. In contrast to the role of α-synuclein in neuronal degeneration, γ-synuclein does not cause neuronal apoptosis (65). γ- and β-Synucleins are not detected in Lewy bodies or Lewy neurites (8, 14), and they are intrinsically less fibrillogenic than α-synuclein and cannot form mixed fibrils with α-synuclein (44). Therefore, it would be of interest to determine whether the protective role of γ-synuclein is lost in the neurons of PD and other neurodegenerative diseases. Overall, our studies provide new insight into the biological function of γ-synuclein and its role in the pathogenesis of the breast and ovary and offer a new therapeutic target for future treatment.

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