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# Tissue Transglutaminase is Not Involved in the Aggregate Formation of Stably Expressed α-Synuclein in SH-SY5Y Human Neuroblastoma Cells

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Intraneuronal deposition containing α-synuclein is implicated in the pathogenesis of synucleinopathies including Parkinsons disease (PD). Although it has been demonstrated that cytoplasmic inclusions of wild type α-synuclein are observed in the brain of PD patients and that α-synuclein mutations such as A30P and A53T accelerate aggregate formation, the exact mechanism by which α-synuclein forms insoluble aggregates is still controversial. In the present study, to understand the possible involvement of tissue transglutaminase (tTG) in aggregate formation of α-synuclein, SH-SY5Y cell lines stably expressing wild type or mutant (A30P or A53T) α-synuclein were created and aggregate formation of α-synuclein was observed upon activation of tTG. The data demonstrated that α-synuclein negligibly interacted with tTG and that activation of tTG did not result in the aggregate formation of α-synuclein in SH-SY5Y cells overexpressing either wild type or mutant α-synuclein. In addition, α-synuclein was not modified by activated tTG *in situ*. These data suggest that tTG is unlikely to be a contributing factor to the formation of aggregates of α-synuclein in a stable cell model.

**Key words:** Tissue transglutaminase (tTG), α-Synuclein, Inclusion, A30P, A53T, SH-SY5Y human neuroblastoma

## INTRODUCTION

Parkinsons disease (PD) is a progressive neurodegenerative disorder characterized by tremor, slowness of movement, rigidity and postural instability (Lotharius and Brundin, 2002). These symptoms are primarily attributable to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (Olanow and Tatton, 1999). Aggregation of α-synuclein and its deposition as fibrils in intracellular inclusions are implicated in the pathogenesis of PD and several other neurodegenerative diseases, including dementia with Lewy bodies (DLB), multiple-system atrophy, and the Lewy body variant of Alzheimer's disease (Galvin *et al.*, 2001). Two autosomal dominant mutations in synuclein (A30P and A53T) cause familial, early-onset PD (Galvin *et al.*, 2001; Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997). α-synuclein is a protein that is highly expressed in neurons, being particularly abundant at pre-synaptic terminals and exists in either a natively unfolded (Weinreb *et al.*, 1996) or as an α-helix in the presence of phopholipid vesicles (Davidson *et al.*, 1998). Because of its unfolded structure, α-synuclein is prone to self-aggregate and to sequester other proteins into Lewy bodies, resulting in the contribution to the pathogenesis of PD (Conway *et al.*, 1998; Giasson *et al.*, 1999; Paik *et al.*, 1998). However, the biochemical and biophysical factors that induce aberrant α-synuclein aggregation are not clearly understood.

The transglutaminases are a family of calcium-dependent enzymes that catalyze the formation of ε-(γ-glutamyl)lysine isopeptide bonds between substrate proteins rendering the resulting cross-linked protein complexes insoluble (Folk, 1983; Green, 1993; Lorand and Conrad, 1984).

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Transglutaminases also catalyze the incorporation of polyamines into substrate proteins (Greenberg *et al.,* 1991; Lorand and Conrad, 1984). Tissue transglutaminase (tTG), expressed in predominantly in neurons (Lesort *et al.,* 1999; Miller and Anderton, 1986), has GTPase and ATPase activities in addition to its transamidating activity (Lai *et al.,* 1998; Zhang *et al.,* 1998b). Recent studies have demonstrated that tTG is involved in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease and Huntingtons disease by catalyzing the formation of insoluble aggregates of causative proteins (Ho *et al.,* 1994; Karpuj *et al.,* 1999; Miller and Johnson, 1995; Selkoe *et al.,* 1982). Although it is clear that cytoplasmic inclusion of α-synuclein is observed in the brain of PD patients, the exact mechanism by which α-synuclein forms insoluble aggregates remains inconclusive (Andringa *et al.,* 2004; Conway *et al.,* 2000; Junn *et al.,* 2003; Wood *et al.,* 1999).

In the present study, SH-SY5Y cell lines stably expressing wild type or mutant α-synuclein constructs were established to determine the potential role of tTG in aggregate formation. Using these cells, we demonstrated that tTG and α-synuclein did not interact and that α-synuclein was not modified by tTG *in situ.* These data demonstrate that tTG is unlikely to be a contributing factor to the formation of aggregates of α-synuclein.

## MATERIALS AND METHODS

### Cell culture and generation of stable cell lines

Wild type and mutant (A30P and A53T) α-synuclein constructs were generous gift from Dr. Yen (Ko *et al.,* 2000). Human neuroblastoma SH-SY5Y cells were transfected by electroporation (Gene Pulser II, BIO-RAD) according to the suppliers instructions. SH-SY5Y cells stably expressing pcDNA3 vector alone, wild type α-synuclein, A30P mutant, or A53T mutant were selected and maintained on Corning dishes in RPMI 1640 medium supplemented with 20 mM glutamine, 10 units/mL penicillin, 100 μg/mL streptomycin, 5% fetal clone II serum, 10% horse serum, and 100 μg/mL G418 (GIBCO). To initiate differentiation, cells were grown in media containing 1% fetal clone II and 4% horse serum containing 20 μM retinoic acid for 5 days. Previous studies have shown that treatment of SH-SY5Y cells with retinoic acid results in a significant increase in tTG expression (Zhang *et al.,* 1998b). All experiments were carried out on subconfluent cultures.

### Immunoblotting

To evaluate the expression levels of α-synuclein, tTG, and tubulin in stable cells, extracts from cells were prepared and quantitatively immunoblotted. Cells were harvested in cold phosphate-buffered saline (PBS), collected by centrifugation, resuspended in a homogenizing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/mL concentration each of aprotinin, leupeptin, and pepstatin), and sonicated on ice. Protein concentrations of the homogenates were determined using the bicinochoninic acid (BCA) method (Pierce) and diluted to a final concentration of 2 mg/mL with 2X reducing stop buffer (0.25 M Tris-HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, and 10% glycerol with bromophenol blue as the tracking dye). Samples (40 mg of protein) were resolved on 16% or 8% SDS-polyacrylamide gels for α-synuclein or tTG and β-tubulin, respectively, and transferred to nitrocellulose. Blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The blots were then incubated with the anti-α-synuclein monoclonal antibody (1:1000, Transduction Laboratories), anti-tTG monoclonal antibody TG100 (1:750, Neomarkers) or with a monoclonal tubulin antibody (1:2000, Covance) in the same buffer overnight at 4°C. The membranes were then washed three times with TBST and incubated with HRP-conjugated goat anti-mouse IgG (1:2,000) for 2 h at room temperature. The membranes were rinsed three times for 30 min with TBST, followed by four quick rinses with distilled water, and developed with the enhanced chemiluminescence method (ECL) (Amersham).

### *In situ* tTG activity assay

Cells were labeled with 2 mM 5-(biotinamido)pentylamine (Pierce), a biotinylated polyamine, for 45 min. To increase intracellular levels of calcium and activate tTG, 2 nM maitotoxin (MTX, Alexis), which activates voltage-sensitive $Ca^{2+}$ channels, was added to the cells. Because tTG is a substrate of calpain (Zhang *et al.,* 1998a) and because MTX activate calpain (Xie *et al.,* 1998), 25 μM *N*-Acetyl-Leu-Leu-Methioninal (Sigma), a calpain inhibitor, was added for 15 min prior to the addition of MTX. Twenty minutes after the addition of MTX the cells were harvested in lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.05% NP40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/mL concentration each of aprotinin, leupeptin, and pepstatin) and sonicated. A particle-free supernatant was prepared from the homogenate by centrifugation at 2000 g for 5 min at 4°C and the protein concentrations were determined. Transglutaminase activity was quantified by measuring the presence of incorporated 5-(biotinamido) pentylamine into proteins by a microplate assay as described by Zhang *et al.* (Zhang *et al.,* 1998a). To visualize the proteins into which the 5-(biotinamido)pentylamine had been incorporated, samples were electrophoresed on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with HRP-conjugated neutravidin (1:2,000, Pierce). The blots were developed as described above.

### Immunoprecipitation

SH-SY5Y cells stably expressing wild type or mutant α-synuclein were labeled with 2 mM 5-(biotinamido) pentylamine and treated with MTX as described above. The cells were subsequently harvested in immunoprecipitation buffer (0.5% NP40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and a 10 μg/mL concentration each of aprotinin, leupeptin, and pepstatin) and sonicated. A particle-free supernatant was prepared from the homogenate by centrifugation at 2000 g for 5 min at 4°C and protein concentrations were determined. Samples containing 200 mg of protein were immunoprecipitated overnight at 4°C with 10 μg of the monoclonal tTG antibody (TG100). Protein G-Sepharose (Pharmacia, 40 μL of beads) that had been washed previously three times with the immunoprecipitation buffer and blocked with egg albumin (1 mg/mL) to decrease nonspecific binding was added, and the incubation continued for 3 h at 4°C. After the precipitates were washed three times with IP wash buffer (1.0% NP40; 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and a 10 μg/mL concentration each of aprotinin, leupeptin, and pepstatin), 40 μL of 2X reducing stop buffer were added to each sample and the samples were placed in a boiling water bath for 15 min before SDS-polyacrylamide gel electrophoresis and immunoblotting. Blots were probed with HRP-conjugated neutravidin (1:2,000, Sigma) and then stripped by incubation in 100 mM b-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 7.6, at 50°C for 30 min with agitation and reprobed with the rabbit anti-synuclein polyclonal antibody (1:1000, Chemicon).

### Coimmunoprecipitation

Cell lysates were prepared as described above, except that the cells were not prelabeled with 5-(biotinamido) pentylamine. Samples containing 200 mg of protein were precleared for 1 h at 4°C with protein G-Sepharose (Pharmacia) that had been washed previously three times with the immunoprecipitation buffer for precipitation of tTG. Precleared samples were immunoprecipitated overnight at 4°C with 1.2 μg of a monoclonal antibody to tTG (CUB7402, Neomarkers). In some experiments nonimmune rabbit IgG was included as a control. Protein G-Sepharose (50 μL of beads) was added and the incubation continued overnight at 4°C. After the precipitates were washed three times with IP wash buffer, 50 μL of Laemmli stop buffer were added to each sample and the samples were placed in a boiling water bath for 15 min before SDS-PAGE and immunoblotting. Blots were probed with the polyclonal α-synuclein antibody and stripped as described above, and reprobed with the monoclonal tTG antibody 4C1.

## RESULTS

To determine the putative role of tTG in aggregate formation of α-synuclein, SH-SY5Y human neuroblastoma cells stably transfected with wild type or mutant (A30P or A53T) α-synuclein constructs were established. Immunoblot analysis revealed that there was an α-synuclein immunoreactive band, as expected, at ~17 kDa in SH-SY5Y cells overexpressing both wild type or mutant α-synuclein (Fig. 1, top panel). As expected, retinoic acid treatment increased the expression of tTG without affecting the expression of the α-synuclein (Fig. 1, middle panel). Both α-synuclein and tTG were cytoplasmic and aggregates of α-synucleins were not observed in immunocytochemical analyses (data not shown).

To determine *in situ* tTG activity in SH-SY5Y cells overexpressing wild type or mutant α-synuclein, 5-(biotinamido) pentylamine was used as a probe for the tTG activity (Zhang *et al.*, 1998b). Because tTG is a calcium-activated enzyme, intracellular levels of calcium were elevated by treating the cells with MTX, and TG activity was measured. Increasing intracellular calcium levels in cell treated with retinoic acid resulted in a significant increase in tTG activity (Fig. 2). The presence of wild type or mutant α-synuclein did not alter the basal activity of tTG or the overall activity of tTG in response to MTX treatment (Fig. 2). In addition, the profile of proteins modified by tTG in the cells did not differ between the cell lines as shown in a representative images (Fig. 2B).

To determine whether tTG and α-synuclein interact, and if increasing the activity of tTG alters this interaction, coimmunoprecipitation studies were carried out. Cells expressing wild type or mutant α-synuclein were incubated

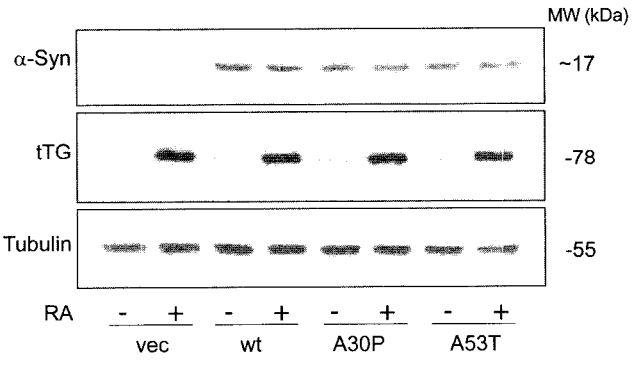


**Fig. 1.** Representative immunoblots of the expression of α-synuclein (α-Syn, top), tTG (middle), and β-tubulin (bottom) in SH-SY5Y cells stably expressing vector (vec), wild type (wt), or mutant (A30P or A53T) of human α-synuclein constructs. Cells were incubated in the absence (-) or presence (+) of 20 μM retinoic acid (RA) for 5 d before immunoblotting. Treatment of the cells with RA resulted in a significant increase in tTG levels, but did not alter the expression or levels of α-synuclein. Positions at which molecular mass standards (kDa) migrate are indicated at the right.

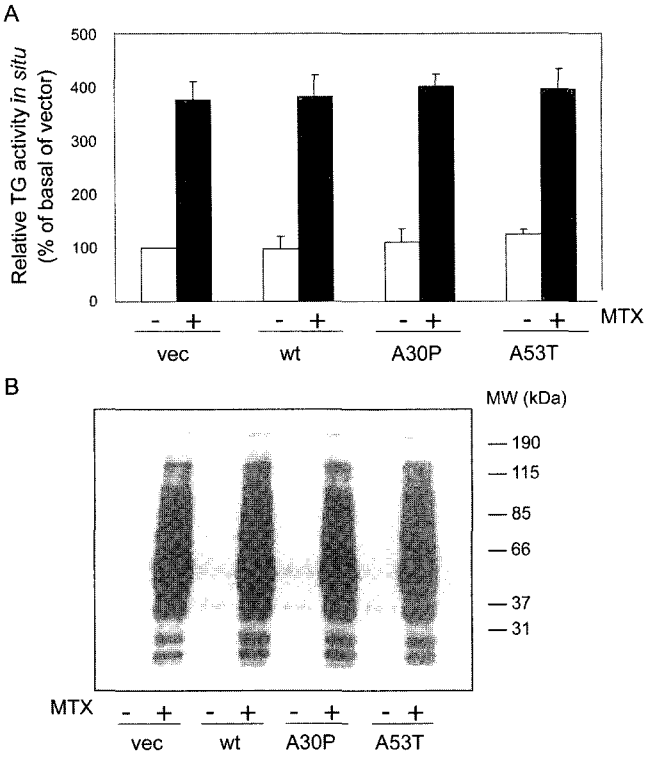


**Fig. 2.** Quantitative (A) and qualitative (B) analysis of the TG-catalyzed incorporation of 5-(biotinamido)pentylamine into proteins as a measure of *in situ* activity. SH-SY5Y cells stably expressing vector (vec), wild type (wt), A30P, or A53T were treated with 20 μM retinoic acid (RA) for 5 days, labeled with 5-(biotinamido)pentylamine, and incubated with 20 min in the absence (-) or presence (+) of 2 nM maitotoxin (MTX). Quantitative analysis of the TG-catalyzed incorporation of 5-(biotinamido)pentylamine into proteins using a microplate-based assay demonstrated that tTG expression induced by RA resulted in significant increases in MTX-stimulated TG activity. Activity was normalized to the basal activity of vec and presented as Mean±SEM (n=3 separate experiments, each in triplicate).

with retinoic acid for 5 days and then incubated in the absence or presence of MTX. Lysates were immunoprecipitated with a monoclonal tTG antibody TG100, probed for α-synuclein with the polyclonal α-synuclein antibody (Chemicon), stripped, and reprobed with a monoclonal tTG antibody (4C1). tTG and synuclein interacted very weakly and activation of tTG did not increase the association between tTG and α-synuclein (Fig. 3). To further confirm this association between tTG and α-synuclein, cell lysates were immunoprecipitated with α-synuclein antibody and then probed with tTG antibody. Identical results showing negligible interaction between the two proteins were obtained (data not shown).

Formation of polymerized α-synuclein upon activation of tTG was observed. Soluble oligomers incompletely migrate in SDS-polyacrylamide gels showing smearing immunoreactivities in upper part of the gel depending on the degree of polymerization. However, immunoreactivities

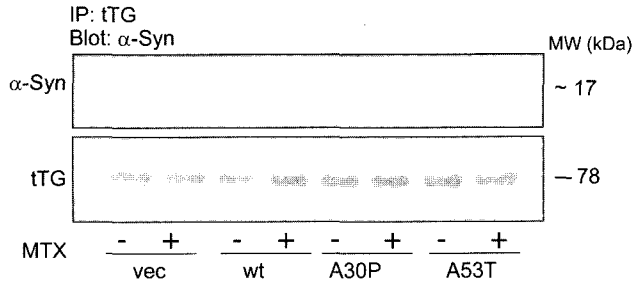


**Fig. 3.** *In situ* association between α-synuclein and tTG in response to MTX treatment. Cells stably expressing wild type, or mutant α-synuclein (A30P or A53T) were treated with 20 μM retinoic acid (RA) for 5 days and incubated for 20 min in the absence (-) or presence (+) of 2 nM MTX. tTG in lysates were immunoprecipitated with the monoclonal tTG antibody (4C1) and the precipitates were immunoblotted with the polyclonal α-synuclein antibody (α-Syn, top panel). The immunoblots were stripped and reprobed with the monoclonal tTG antibody (TG100) (bottom panel). Positions at which molecular mass standards (kDa) migrate are indicated at the right.

of insoluble aggregates, which could not penetrate into separating gels, were observed in stacking gels. To determine the formation of soluble and insoluble inclusions of α-synuclein, cell lysates were immunoblotted with α-synuclein antibody and stacking gel and entire separating gel were examined for soluble and/or insoluble oligomers and polymers of α-synuclein. Dimers and/or polymers of α-synuclein were not detected in SH-SY5Y cells overexpressing either wild type or mutant (A30P and A53T) α-synuclein. Further, activation of tTG did not provide oligomerization of α-synuclein (Fig. 4).

Although tTG does not directly cross-link α-synuclein, it

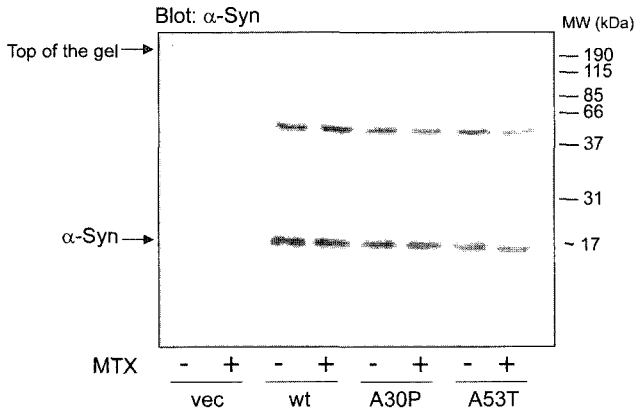


**Fig. 4.** No polymerization of α-synuclein by activated tTG. Cells stably expressing vector (vec), wild type (wt), or mutant α-synuclein (A30P or A53T) were treated with 20 μM retinoic acid (RA) for 5 days and incubated for 20 min in the absence (-) or presence (+) of 2 nM MTX. α-synuclein in lysates were immunoblotted with the polyclonal α-synuclein antibody (Chemicon). Entire blots were examined to detect polymerized α-synuclein (α-Syn). Top of the gel indicates beginning of separating gel. Positions at which molecular mass standards (kDa) migrate are indicated at the right.

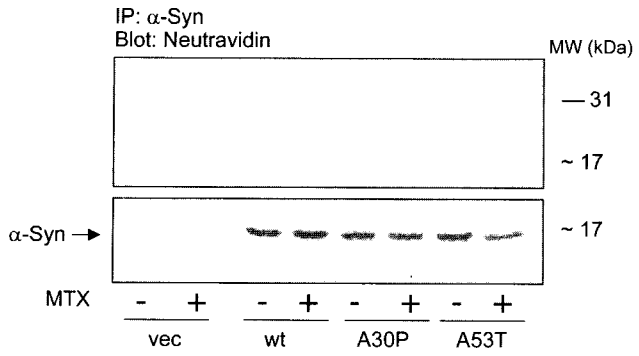


**Fig. 5.** No selective modification of α-synuclein by tTG. Cells stably expressing wild type (wt), or mutant α-synuclein (A30P or A53T) were treated with 20 μM retinoic acid for 5 d, labeled with 5-(biotinamido) pentylamine, and incubated for 20 min in the absence (-) or presence (+) of 2 nM MTX. α-Synuclein in lysates was immunoprecipitated with the polyclonal α-synuclein antibody and the precipitates were immunoblotted with neutravidin-HRP, which recognizes proteins that are modified by tTG (top panel). The immunoblots were stripped and reprobed with the polyclonal α-synuclein antibody (α-Syn, bottom panel). Positions at which molecular mass standard (kDa) migrate are indicated at the right.

is still possible for tTG to incorporate polyamines to α-synuclein resulting in the modulation of biological activity of α-synuclein. The modification of α-synuclein by tTG was examined in SH-SY5Y cells overexpressing wild type or mutant α-synuclein. Cells incubated with retinoic acid were prelabeled with 5-(biotinamido)pentylamine and then incubated in the absence of presence of MTX. Cell lysates were immunoprecipitated with the polyclonal α-synuclein antibody, probed with HRP-conjugated neutravidin to identify proteins modified by tTG, stripped, and reprobed with α-synuclein antibody (Fig. 5). No modification of α-synuclein, either wild type or mutant (A30P and A53T), was observed (Fig. 5). Given the fact that tTG did not efficiently co-immunoprecipitate with α-synuclein (Fig. 3), this finding was not unexpected.

## DISCUSSION

The presence of insoluble aggregates in the brain of PD patients is a characteristic pathological hallmark of the disease although the exact role of α-synuclein aggregates in the pathogenesis of PD remains inconclusive (Lotharius and Brundin, 2002). In the present study, the data demonstrated that tTG did not contribute to the aggregate formation of stably expressed α-synuclein in an SH-SY5Y human neuroblastoma cell model. *In vitro*, recombinant α-synuclein form fibrils that are similar to those found in Lewy bodies (Conway *et al.*, 1998; Giasson *et al.*, 1999; Narhi *et al.*, 1999). This aggregation is accelerated for the pathogenic A30P and A53T α-synucleins, suggesting that accelerated α-synuclein fibril formation may contribute to the early onset of familial PD (Narhi *et al.*, 1999). In the present study, formation of aggregates was not facilitated even in SH-SY5Y cells overexpressing A30P or A53T mutant α-synuclein upon activation of tTG, suggesting that tTG is not a major contributing factor for the aggregate formation of mutant α-synuclein.

Contrary to our results, previous studies have reported that activation of tTG resulted in the formation of insoluble aggregates of wild type α-synuclein (Andringa *et al.*, 2004; Junn *et al.*, 2003). However, those results were obtained with transient expression of α-synuclein, which exhibits temporarily physiologically non-relevant levels of α-synuclein. Aggregates of α-synuclein were not observed in many stable cell models (Kanda *et al.*, 2000; Zourlidou *et al.*, 2003). This phenomenon might be explained due to the relatively low expression levels of α-synuclein in stable cell lines, suggesting that expression levels of α-synuclein is a critical factor for the aggregate formation of α-synuclein along with the mutations. Interestingly, although aggregation was not observed in those stable cell models, α-synuclein mutations such as A30P and A53T showed increased vulnerability to stresses (Kanda *et al.*, 2000; Zourlidou *et al.*, 2003).

Besides the hypothesis that tTG contributes to the aggregate formation of α-synuclein, it has been suggested that aggregate formation by α-synuclein is a nucleation-dependent self-assembly process (Conway *et al.*, 1998; Conway *et al.*, 2000; Krishnan *et al.*, 2003; Volles and Lansbury, 2003). Monomeric α-synuclein oligomerizes to form β-sheet rich protofibrils, which include pore-like structures. The protofibrils act a nucleation-seed to yield amyloid fibrils, which eventually form a Lewy body (Volles and Lansbury, 2003). α-synuclein mutations such as A30P and A53T in familial forms of PD accelerate *in vitro* fibril formation (Conway *et al.*, 1998; Conway *et al.*, 2000). Other factors that contribute to the aggregate formation of α-synuclein have been suggested (Ostrerova-Golts *et al.*, 2000; Paxinou *et al.*, 2001; Sherer *et al.*, 2002). Intracellular nitrative insult such as nitric oxide, superoxide, and peroxinitrite resulted in aggregate formation of α-synuclein in both wild type and mutant cells (Paxinou *et al.*, 2001). Aggregate formation of α-synuclein was also observed with oxidative damages such as rotenone, a mitochondrial complex I inhibitor, and free radical generators including dopamine and hydrogen peroxide (Ostrerova-Golts *et al.*, 2000; Sherer *et al.*, 2002).

In conclusion, the present study demonstrates that activation of tTG does not result in the aggregate formation of stably expressed α-synuclein in SH-SY5Y cells overexpressing either wild type or mutant (A30P and A53T) α-synuclein, suggesting that tTG is unlikely to be a major contributing factor to the formation of insoluble aggregates of α-synuclein.

## ACKNOWLEDGEMENT

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