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Identification of the amino acid sequence motif of α-synuclein responsible for macrophage activation

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ABSTRACT

α-Synuclein (Syn) is implicated in the pathogenesis of PD and related neurodegenerative disorders. Recent studies have also shown that α-synuclein can activate microglia and enhance dopaminergic neurodegeneration. The mechanisms of microglia activation by α-synuclein, however, are not well understood. In this study, we found that not only α-synuclein but also β- and γ-synucleins activated macrophages (RAW 264.7) *in vitro*. Macrophages treated with synuclein proteins secreted TNF-α in a dose-dependent manner. Synuclein family proteins also increased mRNA transcription of COX-2 and iNOS. Two α-synuclein deletion mutants, SynΔNAC and Syn61–140, activated macrophages, while deletion mutants Syn1–60 and Syn96–140 did not significantly activate them. Finally, we demonstrated that macrophage activation by α-synuclein was accompanied by phosphorylation of ERK. These results suggest that synuclein family proteins can activate macrophages, and that macrophage activation needs both the N-terminal and C-terminal domains of α-synuclein, but not the central NAC region.

Introduction

α-Synuclein, an acidic, heat-resistant and unstructured protein of 140 amino acids long, is highly expressed in brain tissues and primarily localized at the presynaptic terminals of neurons [1,2]. α-Synuclein is widely expressed in the central nervous system (CNS). In addition, α-synuclein is expressed in various tissues [2]. α-Synuclein consists of three distinct regions. The N-terminal region (1–60 amino acid residues) contains KTKEGV repeats, which form amphipathic α-helices similar to the lipid binding domain of apolipoproteins. The central region (61–95 amino acids residues) is a very hydrophobic NAC (non Aβ-component of Alzheimer's disease) peptide, and the C-terminal region (96–140 amino acid residues) is primarily composed of acidic amino acids [2,3]. In addition to α-synuclein, β-, γ-synucleins and synoretin, which belong to the synuclein family, have been identified in humans [4–7].

α-Synuclein has traditionally been considered a cytoplasmic protein [1]. This view was challenged recently by the finding that α-synuclein has been detected in extracellular biological fluids, including human cerebrospinal fluid (CSF) and blood plasma in both healthy subjects and patients with Parkinson's disease [8–10]. Recent studies have also shown that γ-synuclein, as well as α-synuclein, is present in CSF. In particular, α- and γ-synucleins are more abundant in aged subjects with neurodegenerative and vascular changes [11]. Moreover, a recent report demonstrated that α-synuclein is rapidly secreted from cells via unconventional, ER/Golgi independent exocytosis [12], suggesting that extracellular α-synuclein may function physiologically or pathologically. In agreement with these suggestions, it has been recently reported that extracellular α-synuclein activates microglia, THP-1 cells and astrocytes [13–16]. Extracellular α-synuclein also induces microglial phagocytosis [17].

In the present study, we investigated the molecular mechanisms by which α-synuclein activates macrophages. To identify the amino acid sequence motif of α-synuclein responsible for macrophage activation, we used synuclein family proteins and several types of α-synuclein deletion mutants. We compared their influence on tumor necrosis factor-alpha (TNF-α) secretion, and looked at Cytochrome c oxidase subunit 2 (COX-2) and inducible Nitric oxide synthase (iNOS) expression as macrophage activation markers. We also investigated the downstream signaling of α-synuclein-induced macrophage activation.

Materials and methods

Materials. Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were obtained from Gibco-Invitrogen

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(Carlsbad, CA). Lipopolysaccharide (LPS) and Polymyxin B sulfates were purchased from Sigma (St. Louis, MO). ECL solution and BCA protein assay kits were purchased from PIERCE (Rockford, IL). Antibodies to P44/42 mitogen activated protein kinase (MAPK), phospho-P44/42 MAPK, Stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), phospho-SAPK/JNK, p38 MAPK and phospho-p38 MAPK were obtained from Cell Signalling Technology (Danvers, MA). Antibody against α-tubulin was obtained from Sigma (St. Louis, MO). The ELISA TNF kit was from BD science (555212 and 558874, Franklin Lakes, NJ). All other reagents used in this study were analytical grade and obtained from either Sigma (St. Louis, MO) or USB (Cleveland, OH).

Protein expression and purification. Synuclein proteins were overexpressed in E. coli (Bl21), and the recombinant proteins were purified as described previously [18]. Briefly, the transformed bacteria were grown in LB medium with 0.1 mg/ml ampicillin at 37 °C to an A600 of 0.8, and then cultured for an additional 4 h after being induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 8000*g* for 10 min, resuspended in 20 mM MES pH 6.0, and then disrupted by ultrasonication. The supernatant was purified with DEAE anion-exchange chromatography, followed by CM cation-exchange chromatography in 20 mM MES, pH6.0. The bound proteins were eluted with a linear gradient between 0.1 M and 0.5 M NaCl at a flow rate of 1.5 mL/min. All proteins were further purified on an FPLC gel-filtration column (GE healthcare, Sweden) pre-equilibrated with PBS, pH7.4. All proteins were concentrated and buffer changed with a Centricon apparatus (Satorius Stedim Biotech, Germany). Proteins were quantitated with the BCA assay, then filtered and stored at 4 °C until use.

Cell culture. RAW 264.7, a mouse macrophage cell line obtained from the Korean Type Culture Collection, was grown in DMEM with 10% FBS and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

ELISA. RAW 264.7 cells (1×10^5 per well) were cultured in 24 well plates overnight. The medium was then removed and replaced with fresh DMEM containing 10% FBS. Cells were incubated with the indicated doses of LPS or indicated doses of recombinant protein with 10 μg/ml polymyxin B sulfate for 18 h. The cell-free supernatants were then harvested, and the TNF-α levels in the supernatants were determined using ELISA kits according to each manufacturer's instructions.

Activation of human primary macrophages. Peripheral blood mononuclear cells (PBMC) from healthy volunteers were obtained through passage over Ficoll-hypaque gradient (GE Healthcare, Sweden). The cells were then washed and resuspended in RPMI 1640 medium with 10% FBS at a concentration of 4×10^6 cells/ml. These cells were placed on 24 well plates, at 1ml/well, and incubated for 2 h at 37 °C in 5% CO_2. Nonadherent cells were removed from the plates, and adherent cells were cultured in RPMI 1640 medium with 10% FBS for 4 days. Cells were incubated with the indicated doses of LPS or 5 μM of recombinant synuclein proteins with 10 μg/ml polymyxin B sulfate for 24 h. The cell-free supernatants were then harvested, and the TNF-α levels in the supernatants were determined using ELISA kits according to each manufacturer's instructions.

RT-PCR. RAW 264.7 cells (2×10^6) were incubated with the indicated doses of proteins for 6 h and then harvested. Total RNA was isolated using an RNeasy mini kit (Qiagen, Santa Clara, CA). To synthesize cDNA, 1 μg of each RNA sample was mixed with 100 ng of random hexamers, 6 μl of 5× first strand buffer, 12 μl of 2.5 mM dNTPs (TaKaRa, Shiga, Japan) and 200 units of murine Molony leukemia virus reverse transcriptase (Invitrogen) and incubated at 42 °C for 80 min. The reaction mixture was boiled at 95 °C for 5 min. cDNA was amplified by PCR using PCR PreMix (Bioneer, Seoul, Korea) and a pair of primers specific for the genes of interest. PCR was performed using the following specific oligonucleotide primer sets: COX-2 forward, 5′-TTC TTC AAC CTC TCC TAC TAC-3′, and reverse, 5′-GCA CGT AGT CTT CGA TCA CTA-3′; iNOS forward, 5′-ATG TCC GAA GCA AAC ATC ACA-3′, and reverse, 5′-TAA TGT CCA GGA AGT AGG TGA-3′; TNF-α forward, 5′-CTA CTG AAC TTC GGG GTG ATC-3′, and reverse, 5′-CAG TCG GCT AAA CGA TAG AGT-3′; GAPDH forward, 5′-GAT CAT CAG CAA TGC CTC CTC-3′, and reverse, 5′-TGT GGT CAT GAG TCC TTC CA-3′.

Western blot analysis. RAW 264.7 cells (5×10^6) were incubated with the indicated doses of proteins for the indicated times and then harvested. The cells were lysed with lysis buffer (10 mM Tris–HCl, pH7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 15 μg/ml leupeptin, 2 mM NaF and 2 mM $NaVO_4$). The protein bands were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Pall Corporation, Ann Arbor, MI, USA). The membranes were blocked with blocking buffer [Tris-buffered saline (TBS) containing 5% nonfat dried milk] for 2 h and incubated with the indicated primary antibodies for 1 h. After washing three times with TBS containing 0.1% Tween 20 (TBS-T), the membranes were incubated with secondary antibodies for 1 h. After washing three times with TBS-T, the membranes were developed using an ECL kit and then exposed to FUJI X-ray film (Tokyo, Japan).

Results

Synuclein family proteins can induce TNF-α secretion in RAW 264.7 cells

To elucidate whether synuclein proteins activate macrophages, RAW 264.7 murine macrophage cells were first incubated with α-synuclein, and then the secretion of TNF-α in the cells was analyzed by ELISA. We used polymyxin B to rule out the effect of contaminating endotoxin in the solution of recombinant proteins. When cells were incubated with 10 μg/ml polymixin B, 10 ng/ml LPS-induced TNF-α secretion was completed inhibited (Fig. 1A), indicating that polymixin B treatment could inhibit the endotoxin contamination in recombinant proteins. Under these conditions, α-synuclein induced TNF-α secretion in RAW 264.7 cells in a dose-dependent manner (Fig. 1A). To rule out the effect of cell death by α-synuclein, we also performed an MTT assay, and observed that α-synuclein did not induce cell death of RAW 264.7 cells under the same conditions (data not shown).

We next used β- and γ-synucleins to clarify whether the effect of α-synuclein on TNF-α secretion in RAW 264.7 cells was a general property of synuclein family proteins. As shown in Fig. 1, β- and γ-synucleins, like α-synuclein, induced TNF-α secretion in RAW 264.7 cells in a dose-dependent manner (Fig. 1B and C). All synuclein proteins appeared to induce similar amounts of TNF-α secretion in RAW 264.7 cells. We also observed that α-synuclein enhanced TNF-α secretion in BV-2 microglia cells (data not shown). Furthermore, when cells were incubated with α-, β- and γ-synucleins, all three synuclein proteins induced the expression of TNF-α, COX-2 and iNOS mRNAs (Fig. 1D).

Effects of α-synuclein point mutants A30P, E46K and A53T on the induction of TNF-α secretion in RAW 264.7 cells

The three point mutants of α-synuclein (A30P, E46K and A53T) that are associated with a few cases of familial Parkinson's disease [19–21] have been thoroughly studied to determine the role of α-synuclein in the pathogenesis of Parkinson's disease. These mutant forms of α-synuclein appear to have different properties from the wild-type α-synuclein with respect to aggregation patterns, binding to lipid membranes and toxicity to cells [22–24]. We investigated whether these α-synuclein point mutants might function differently in the activation of macrophages. When RAW 264.7 cells were incubated with α-synuclein point mutants, each mutant

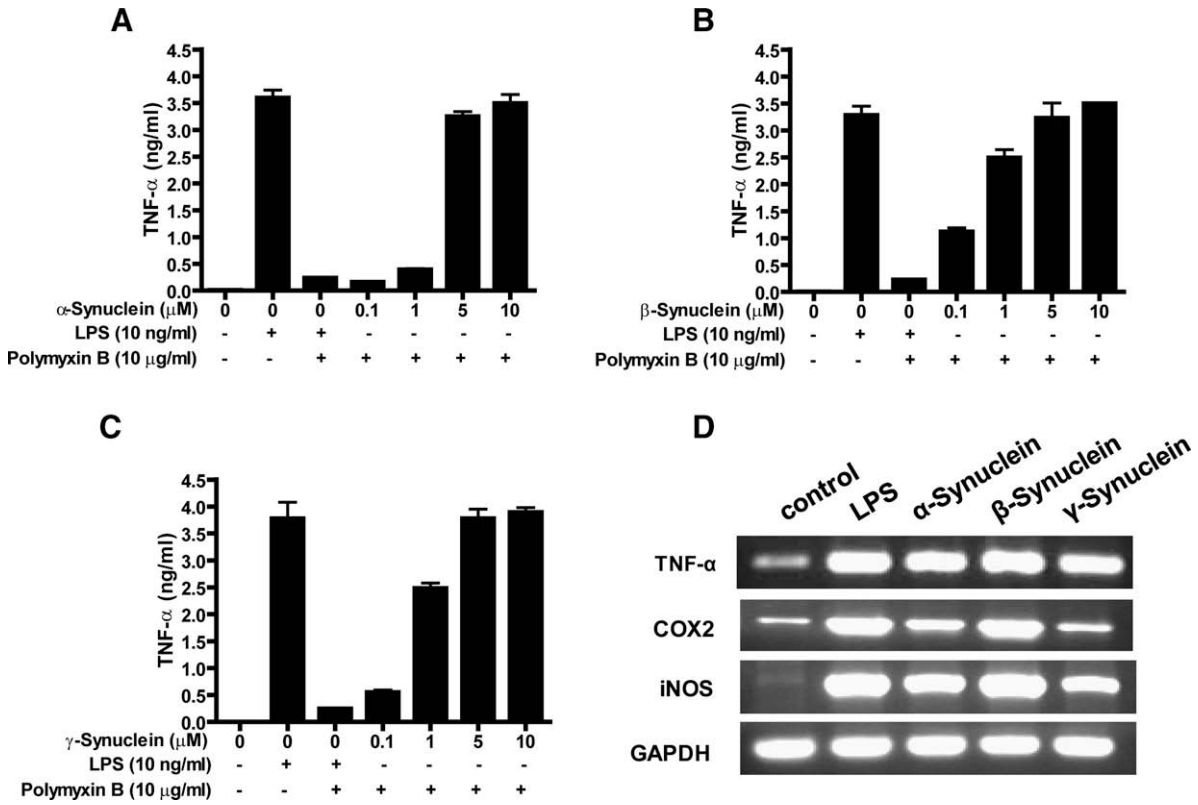

Fig. 1. Synuclein family induced TNF-α secretion and the expression of TNF-α, COX-2 and iNOS in RAW 264.7 cells. RAW 264.7 cells were incubated with the indicated amounts of (A) α-, (B) β- or (C) γ-synuclein for 18 h. Aggregated forms of α-synuclein were removed by gel-filtration chromatography before use. LPS was used for a positive control, and the effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment. The amount of secreted TNF-α was measured in cell-free supernatants by ELISA. (D) RAW 264.7 cells were treated with 1 μM of each synuclein family protein for 6 h. Total RNA was extracted from the treated cells and subjected to RT-PCR to quantify the mRNA transcripts of TNF-α, COX-2 and iNOS. The internal control was set by GADPH. The data are presented as a mean of at least three independent experiments (mean ± SD).

induced the secretion of TNF-α in the cells in a dose-dependent manner (Fig. 2A–C), suggesting that these point mutations do not affect the mechanism by which wild-type α-synuclein stimulates RAW 264.7 cells to secrete TNF-α. Among these point mutants, A53T appeared to induce more TNF-α secretion, particularly at low doses of synuclein treatment (Fig. 2C).

Effects of α-synuclein deletion mutants on the induction of TNF-α secretion in RAW 264.7 cells and primary macrophages

To elucidate the region of α-synuclein responsible for the induction of TNF-α secretion in RAW 264.7 cells, we used four deletion mutants: Syn1-60, containing the N-terminal region; SynΔNAC, containing only the N- and C-terminal regions; Syn61–140, containing NAC and the C-terminal region and Syn96–140, containing only the C-terminal region of α-synuclein (Fig. 3A). When RAW 264.7 cells were incubated with these α-synuclein deletion mutants, SynΔNAC and Syn61–140 induced the secretion of TNF-α in the cells (Fig. 3C and D), while Syn1–60 and Syn96–140 did not (Fig. 3B and E). Similar results were obtained when human primary macrophages were treated with these α-synuclein deletion mutants. (Fig. 3F). These results suggest that α-synuclein-induced TNF-α secretion requires the C-terminal domain of α-synuclein, as well as the N-terminal domain, which was identified as the internalization domain of α-synuclein in our previous report [18].

Phosphorylation of extracellular signal-regulated kinases (ERK) in synuclein family-mediated RAW 264.7 cell activation

Macrophages can be activated by α-synuclein, and the activated macrophages secrete TNF-α, IL-1β and other inflammatory molecules [15,16]. Inflammatory molecules are induced via the activation of MAP kinase signaling pathways [25]. In particular, the ERK pathway is activated by α-synuclein treatment in microglia and astrocytes [15,16]. We investigated whether MAP kinases were phosphorylated by synuclein family proteins. RAW 264.7 cells were treated with 5 μM each of α-, β- or γ-synuclein for 30 min. Western blot analysis with specific antibodies against phosphorylated MAP kinases showed that total ERK, JNK and p38 expression did not change after synuclein treatment (Fig. 4A–C). Among these kinases, the phospho-ERK signal was significantly greater after synuclein treatment compared with the control group (Fig. 4A). Unlike ERK phosphorylation, however, phospho-JNK and phospho-p38 were not detected (Fig. 4B and C).

Discussion

α-Synuclein has recently been detected in extracellular biological fluids, including CSF and blood plasma in both healthy subjects and patients with Parkinson's disease [8–10], although it remains controversial whether the level of α-synuclein correlates with disease progression. In addition, γ-synuclein was also recently found in CSF, and there is an elevation of both α- and γ-synuclein in CSF from elderly individuals with Alzheimer's disease, Lewy body disease and vascular dementia compared to normal controls [11]. Together with our findings, these data suggest that synuclein family proteins may be generally associated with neurodegeneration followed by immune system activation.

The N-terminal amphipathic regions of synuclein family members are well conserved, but the C-terminal acidic tails are very diverse in both size and sequence [2]. Based on the primary structural features, we speculated that the N-terminal region of α-synuclein

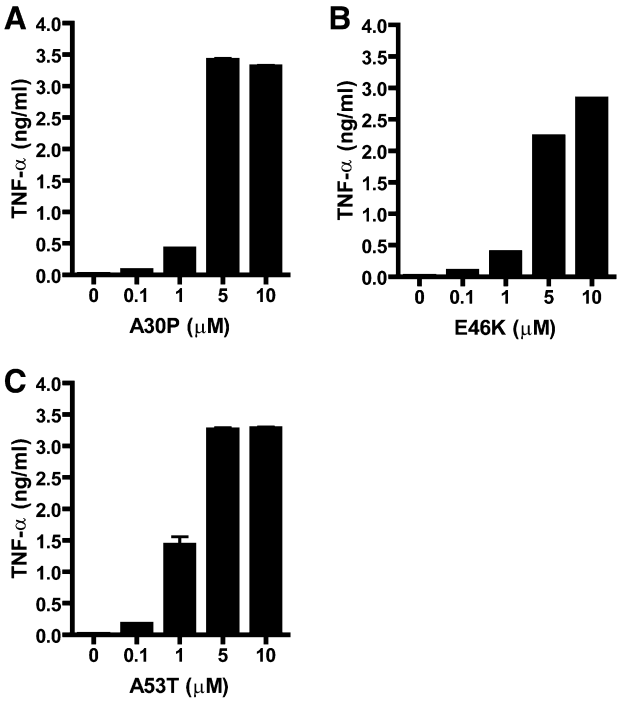

Fig. 2. α-Synuclein point mutants A30P, A53T and E46K induced TNF-α secretion in RAW 264.7 cells. RAW264.7 cells were incubated with the indicated amounts of (A) A30P, (B) E46K and (C) A53T mutant proteins for 18 h. Aggregated forms of α-synuclein point mutants were removed by gel-filtration chromatography before use. The effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment. The amount of secreted TNF-α was measured in cell-free supernatants by ELISA. The data are presented as a mean of at least three independent experiments (mean ± SD).

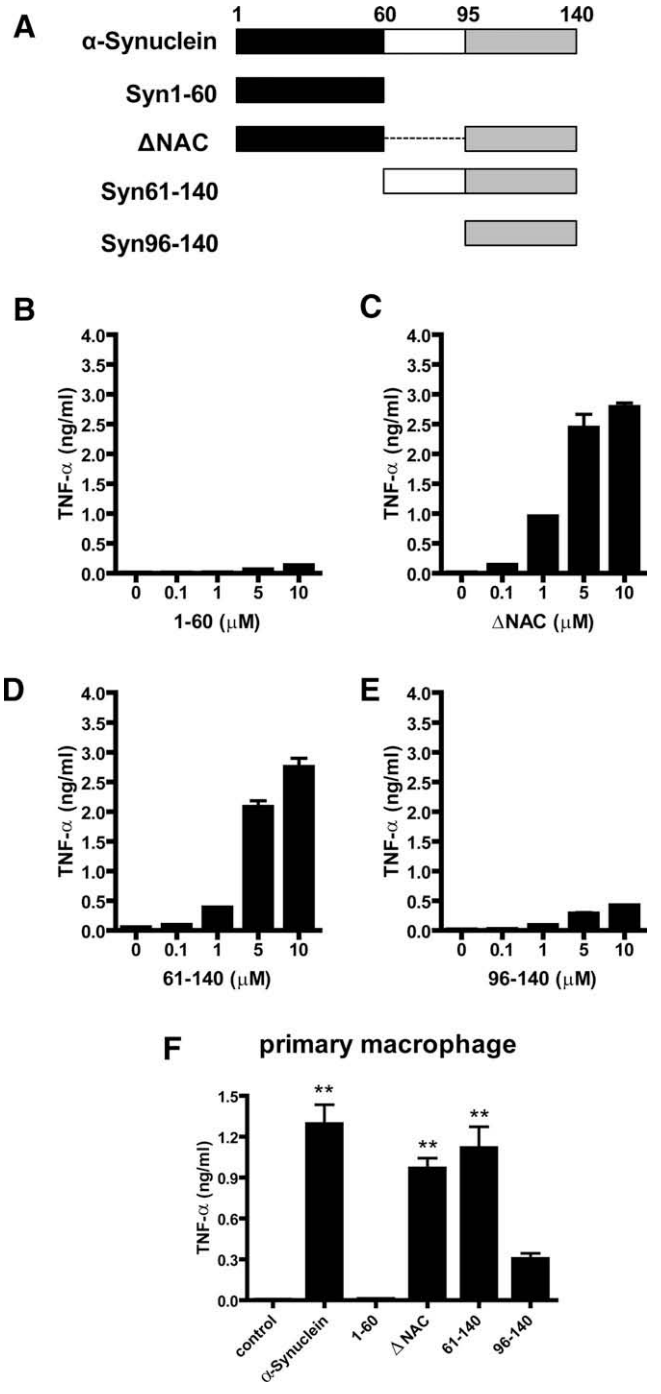

Fig. 3. Effects of α-synuclein deletion mutants Syn1–60, ΔNAC, Syn61–140 and Syn96–140 on the induction of TNF-α secretion in RAW 264.7 cells. RAW 264.7 cells were incubated with the indicated amounts of (B) Syn1–60, (C) ΔNAC, (D) Syn61–140 and (E) Syn96–140 for 18 h. (F) Primary macrophage cells were incubated with 5 μM of α-synuclein, Syn1–60, ΔNAC, Syn61–140 and Syn96–140 for 24 h. Aggregated forms of α-synuclein deletion mutants were removed by gel-filtration chromatography before use. The effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment, and the amount of secreted TNF-α was measured in cell-free supernatants by ELISA. The data are presented as a mean of at least three independent experiments (mean ± SD). Statistical significance was evaluated using one-way ANOVA with post test. $^{**}p < 0.01$ against control.

could be important in RAW 264.7 cell activation. Interestingly, however, α-synuclein deletion mutants SynΔNAC and Syn61–140 activated RAW 264.7 cells and primary macrophages, while Syn1–60 and Syn96–140 did not. The effect of ΔNAC is consistent with a report that the α-synuclein deletion mutant Δ71–82, which is unable to aggregate due to the lack of a corresponding middle hydrophobic region, effectively stimulates THP-1 cells [15]. We previously found that the N-terminal KTKEGV repeat of α-synuclein is essential for its penetration into cells [18]. Based on these findings, it may be that the 1–60 or NAC region of α-synuclein acts as a carrier that transports α-synuclein into cells, and the region responsible for activating macrophages is the acidic C-terminal. Although the C-terminals of α-, β-, and γ-synucleins have low sequence homology, they do share the common property of containing many acidic amino acids. The C-terminal regions of α-, β-, and γ-synucleins are also capable of providing heat-resistance to other proteins [26]. Although we could not exclude the possibility that α-synuclein may bind cell surface receptors and transduce signals for the activation of macrophages, our results suggested that penetration by α-synuclein may be essential for macrophage activation. The CD36 scavenger receptor is associated with α-synuclein-induced microglial activation. Furthermore, Mac-1 is also linked to mutant α-synuclein-induced microglial activation. It seems possible that α-synuclein could activate microglia via direct binding to these receptors. Further study is needed to elucidate the exact mechanism of how synuclein family proteins activate immunocompetent cells.

In summary, we showed that α-, β- and γ-synuclein activated macrophages. The penetration of α-synuclein into the cell may be needed to activate macrophages. The 1–60 or NAC region of α-synuclein may act as a carrier to transport α-synuclein into cells, and the C-terminal acidic region may be responsible for activating macrophages by interacting with other effector molecule(s). Elucidating the mechanisms of immunocompetent cell activation induced by α-synuclein will provide new therapeutic targets for Parkinson's disease.

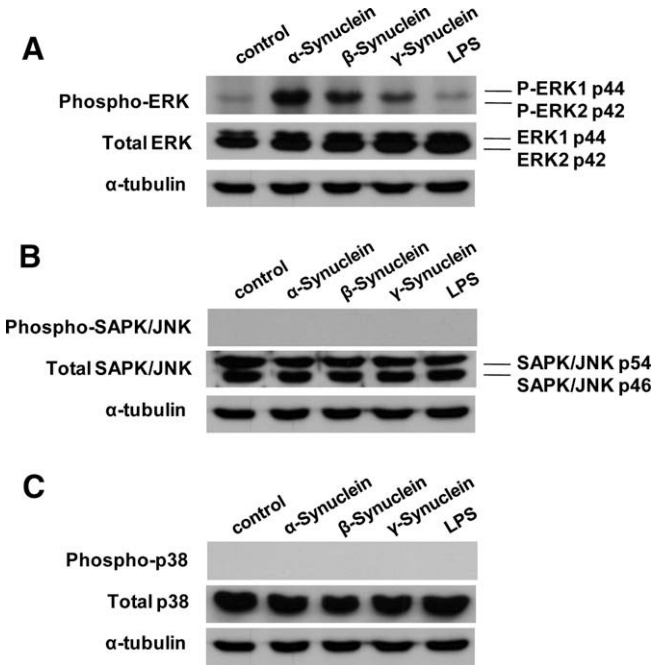

Fig. 4. Phosphorylation of MAP kinases by synuclein family proteins. RAW 264.7 cells were treated with 5 μM of each synuclein family protein for 30 min. Total proteins were extracted from synuclein family protein-treated cells and 70 μg of each extraction were loaded onto SDS–acrylamide gels, followed by western blotting. The internal control was set by α-tubulin. (A) ERK and phospho-ERK, (B) JNK and phospho-JNK and (C) p38 and phospho-p38. The data are presented as one of at least three independent experiments.

Acknowledgments

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