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The BAG2 protein stabilises PINK1 by decreasing its ubiquitination

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ABSTRACT

Mutations in the PTEN-induced putative kinase 1 (PINK1) gene cause an autosomal recessive form of Parkinson disease (PD). Thus far, little is known about what can regulate the ubiquitin proteasome pathway of PINK1. Here, we report BAG2 (Bcl-2-associated athanogene family protein 2), a member of the BAG family, which directly binds with and stabilises PINK1 by decreasing its ubiquitination. Moreover, we found that BAG2 also binds with the pathogenic R492X PINK1 mutation directly and more tightly. Moreover, BAG2 stabilises the R492X PINK1 mutation by decreasing its ubiquitination to a greater extent than the wild-type species. Our data correlate BAG2 to PINK1 for the first time, strengthening the important role of BAG2 in PD-related neurodegeneration.

1. Introduction

Parkinson disease (PD) is a common movement disorder that is caused by the degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNc) and a more widespread neuronal loss in other brain regions. Genetic research has provided much insight into the molecular mechanisms of this disease [1–7]. Genetic mutations have led to a range of different pathological effects in neurons, such as protein aggregation, proteasomal stress, oxidative stress and mitochondrial impairment. Several mutations in PTEN-induced kinase 1 (PINK1 or PARK6) have been reported to be associated with autosomal recessive, early onset PD, and PINK1 is considered the second most common virulence gene besides Parkin [5,8,9].

The PINK1 protein provides the first evidence of a direct link between the dysfunction of a mitochondrial protein and PD. Previous studies have reported much information about the downstream proteins of PINK1. For example, PINK1/Parkin mediates mitophagy, and PINK1 acts upstream of parkin [10–13]. PINK1 also regulates the mitochondrial protease HtrA2 independent of Parkin [14,15]. Therefore, it is important to stabilise PINK1. However, there are few studies regarding what protein can alter the stability of PINK1. As reported, PINK1 is degraded by the ubiquitin proteasome pathway, and DJ-1wt enhances PINK1 stability [16,17]. Therefore, which other protein can regulate the degradation of PINK1 remains an intriguing question.

The Bcl-2 associated athanogene (BAG) family is a multifunctional group of proteins characterised by the BAG domain (BD), which mediates direct interaction with the ATPase domain of Hsp70/Hsc70 molecular chaperones [18]. As one of the six human BAG proteins, BAG2 can bind with Hsp70 [19] and modulate the paired helical filament Tau levels in neurons through caubiquitin-independent pathway [20]. Furthermore, BAG2 prevents unregulated ubiquitylation of misfolded proteins in a cochaperone-dependent regulatory mechanism [21,22].

In our study, we identified a direct interaction between BAG2 and PINK1, and further investigation revealed that BAG2 prevented the degradation of both wild-type PINK1 and the R492X PINK1 mutation in the ubiquitin proteasome system, with the R492X PINK1 mutation to a remarkably greater extent. These results suggested that BAG2 could stabilise PINK1, especially the R492X PINK1 mutation, which may play an important role in the pathogenesis of PD.

2. Materials and methods

2.1. Antibodies, reagents and plasmids

The following antibodies were used for immunoblotting: HA mouse monoclonal antibody (1:1000, Santa Cruz), UB mouse

Abbreviations: PD, Parkinson disease; PINK1, mutations in PTEN-induced kinase 1; UPS, ubiquitin proteasome system; BAG, Bcl-2associated athanogene; BAG2, Bcl-2-associated athanogene 2; DMEM, Dulbecco's modified Eagle's medium.

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monoclonal antibody (1:500, Santa Cruz), GAPDH mouse monoclonal antibody (1:2000, Chemicon). HA rabbit monoclonal antibody (Abcam) was used for immunoprecipitation. Full length BAG2 cDNA was amplified from a human foetal brain library (Invitrogen) using the forward primer 5′-cgggatccccatggctcaggcgaagatc-3′ and reverse primer 5′-ccgctcgagtaattgaatctgctttcag-3′ (IntroGene) and inserted into the pGEX-5x-1 vector. To construct pEGFP-N1-BAG2, full length BAG2 cDNA was excised from pGEX-5x-1-BAG2 with BamHI/XhoI and subcloned into the pEGFP-N1 vector. The mammalian expression plasmid pKH3-HA-PINK1 was a kind gift from Dr. Bin Li (University of Science & Technology of China).

2.2. Cell culture

The HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% foetal bovine serum (FBS, Hyclone) and penicillin/streptomycin (Invitrogen) in a 5% CO_2 humidified incubator at 37 °C. The cells were transfected with Lipofectamine 2000 (Invitrogen) and the protein levels were analysed by Western blotting.

2.3. Immunofluorescence

The HEK293 cells were grown for 16 h prior to transfection in 12-well plates. The transfections were carried out using plasmid DNA and lipo2000 in 1 ml of OptiMem according to the manufacturer's instructions. After 6 h, the medium was replaced and 1 ml DMEM with 10% FBS was added. After 24 h, the cells were washed with 1×PBS and fixed in 4% paraformaldehyde/PBS for 20 min at ambient temperature. After three 1×PBS washes, the cells were permeabilised by incubation in 1×PBS containing 1% Triton-X100 and 1% BSA for 1 h. The cells were incubated for 2 h at room temperature with the primary mouse monoclonal anti-HA antibody (1:500) resuspended in 1×PBS. After three 1×PBS washes, the primary antibodies were visualised using a 1-h incubation with the appropriate donkey anti-mouse antibody. Then, after three 1×PBS washes, the cells were visualised under the microscope.

2.4. Co-immunoprecipitation

The HEK293 cells were transfected with 4 μl of each plasmid. 24 h later, the cells were washed in cold 1×PBS and harvested in immunoprecipitation buffer. The lysate was precleared for 1 h at 4 °C with 25 μl of protein G (Sigma) and centrifuged at 14,000 rpm. The supernatant was incubated with 5 μl of anti HA antibody or anti EGFP antibody and 60 μl of protein G and rocked at 4 °C overnight. The protein G beads were pelleted and washed with immunoprecipitation buffer. The precipitates were resolved by SDS–PAGE gel and analysed by Western blot.

2.5. GST pull down assays

The GST fusion constructs (GST and GST-BAG2) were transformed into *Escherichia coli* JM109 cells. The GST fusion proteins were incubated with glutathione Sepharose 4B (KPL company) in 1×PBS for 1 h at 4 °C with gentle rocking. The beads were then washed twice with HNTG. To test for direct binding between proteins, the beads were incubated with His-PINK1 or His-PINK1-R492X for 2 h at 4 °C with gentle rocking. The samples were washed five times with HNTG, separated by SDS–PAGE and analysed by Western blot.

2.6. Statistics

All experiments were repeated three times and the data were expressed as the mean ± s.d. from a representative experiment. The data were analysed using ImageJ and SPSS (V16.0, IBM, Chicago, USA). The statistical significance of the differences between the group means was analysed by an ANOVA test where $P < 0.05$ was considered statistically significant.

3. Results

3.1. Colocalisation of BAG2 with PINK1 and the R492X PINK1 mutation in vivo

To examine the subcellular localisation, we co-transfected EGFP-N1 or EGFP-BAG2 with HA-PINK1 in HEK293 cells. EGFP-N1 and EGFP-BAG2 were diffusively distributed in the cytoplasm and EGFP-BAG2 exhibited a colocalisation with HA-PINK1 in cultured HEK293 cells (Fig. 1A). Then, we co-transfected EGFP-N1 or EGFP-BAG2 with the HA-PINK1-R492X mutation in HEK293 cells and EGFP-BAG2 exhibited a more obvious colocalisation with the HA-PINK1-R492X mutation in cultured HEK293 cells (Fig. 1A).

3.2. BAG2 interacts directly with PINK1 and the R492X PINK1 mutation

To investigate the relationship between BAG2 and PINK1, co-immunoprecipitation experiments were performed. We coexpressed EGFP-N1 or EGFP-BAG2 with HA-PINK1 in HEK293 cells, which was followed by immunoprecipitation with EGFP rabbit monoclonal antibody and Western blotting with HA mouse monoclonal antibody. The results showed that BAG2 interacts with PINK1 (Fig. 1B). We also coexpressed EGFP-N1 or EGFP-BAG2 with the HA-PINK1-R492X mutation in HEK293 cells, and the result also showed that BAG2 interacts with the HA-PINK1-R492X mutation. Moreover, protein concentrations in the supernatant were not determined by BCA assay in this qualitative experiment.

To determine whether there is a direct interaction between BAG2 and PINK1/PINK1-R492X, we performed in vitro interaction assays using GST, GST-BAG2, His-PINK1 and His-PINK1-R492X. Consistent with the co-immunoprecipitation results, BAG2 directly bound to PINK1 and the PINK1-R492X mutation (Fig. 1C). Protein concentrations in the supernatant were also not determined by BCA assay in this qualitative experiment.

3.3. BAG2 stabilises PINK1 and the R492X PINK1 mutation

To investigate the interaction between BAG2 and PINK1, we co-transfected EGFP-N1 or EGFP-BAG2 with HA-PINK1 in HEK293 cells. The overexpression of EGFP-BAG2 in HEK 293 cells resulted in an increase in HA-PINK1 as detected by HA mouse monoclonal antibody. At the same time, we used MG132, a proteasome inhibitor, to inhibit proteasomal degradation and found that there was no obvious distinction in PINK1 between the groups (Fig. 2A).

We also coexpressed EGFP-N1 or EGFP-BAG2 with the HA-PINK1-R492X mutation in HEK293 cells. The results showed a marked increase in HA-PINK1-R492X, which was more obvious than the PINK1 group in the absence of MG132. These studies also showed no distinction with MG132 (Fig. 2B).

3.4. BAG2 decrease the ubiquitination of PINK1 and the R492X PINK1 mutation

To ascertain whether BAG2 affects PINK1 and the HA-PINK1-R492X mutation through the ubiquitin proteasome system, we co-transfected EGFP-N1 or EGFP-BAG2 with HA-PINK1 in HEK293 cells, which was followed by immunoprecipitation with HA rabbit monoclonal antibody. In the EGFP-BAG2 group, an HA-PINK1 immuno-complex was brought down by the HA antibody and the ubiquitination of HA-PINK1 was reduced (Fig. 3).

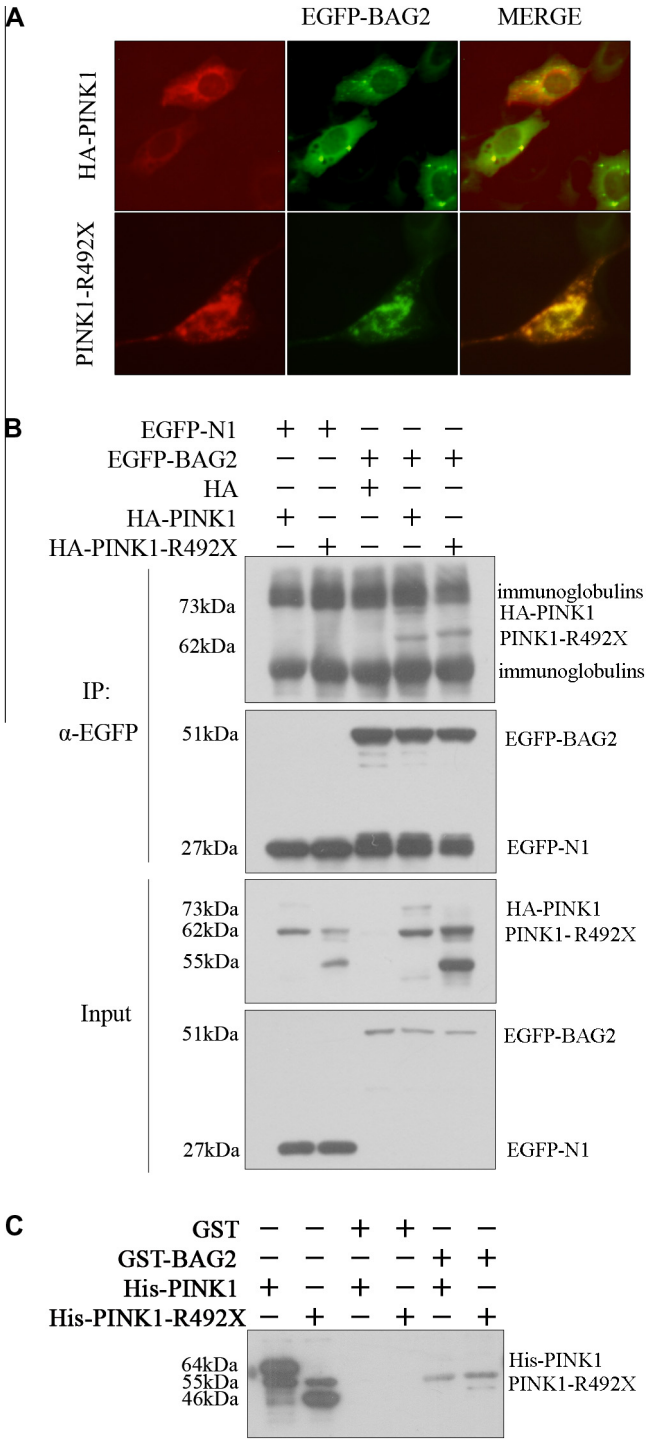

Fig. 1. BAG2 interacted with PINK1 and the R492X PINK1 mutation in vitro and in vivo. (A) Co-localisation of BAG2 with PINK1 and the R492X PINK1 mutation in vivo. The HEK293 cells were transfected with EGFP-N1 (green) or EGFP-N1-BAG2 (green) with HA-PINK1 (red) or HA-PINK1-R492X (red). Then, 24 h after transfection, the merged green and red images showed partial co-localisation of BAG2 with PINK1 or PINK1-R492X in vivo. (B) BAG2 interacts with PINK1 and the R492X PINK1 mutation in vivo. There were five groups expressing EGFP-N1 or EGFP-N1-BAG2 together with HA-PINK1 or HA-PINK1-R492X. Then, 24 h after transfection, the cell lysates was subjected to IP with anti-HA rabbit antibody. The IP and input lysates were analysed by immunoblotting with anti-HA mouse antibodies. The results showed that BAG2 interacts with both wild-type PINK1 and PINK1-R492X in cells. (C) BAG2 directly interacts with PINK1 and the R492X PINK1 mutation in vitro. GST, GST-BAG2, His-PINK1 and His-PINK1-R492X were expressed in *E. coli*, a large quantity of protein was extracted and pull down experiments were performed. There were five groups and the first three groups were negative controls. The results showed that GST-BAG2 could combine with His-PINK1 and His-PINK1-R492X in vitro. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

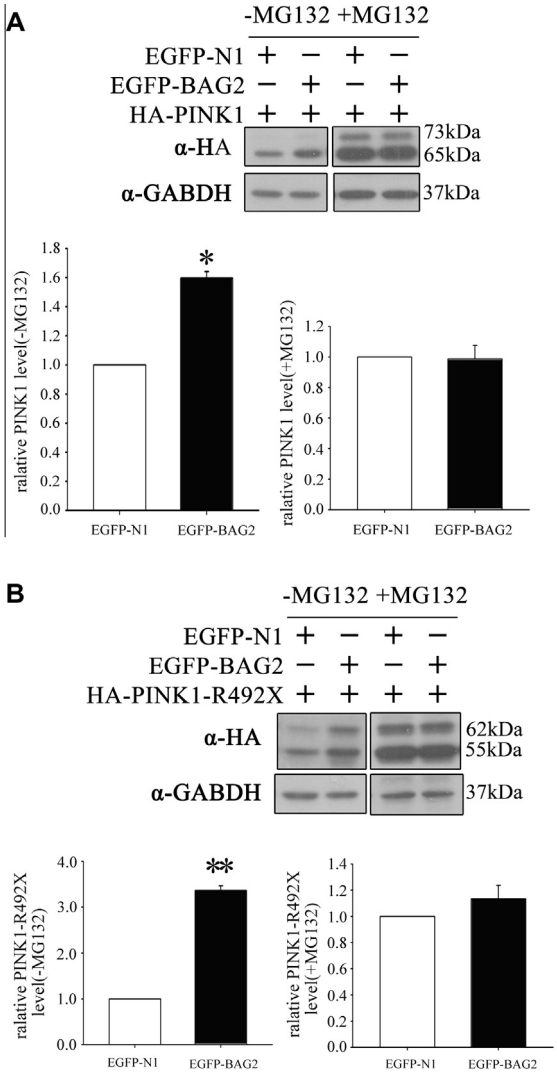

Fig. 2. BAG2 stabilised PINK1 and the R492X PINK1 mutation. (A) BAG2 stabilised PINK1. HEK293 cells were co-transfected with EGFP-N1 or EGFP-N1-BAG2 together with HA-PINK1, with or without MG132 treatment. The PINK1 levels were increased in the EGFP-BAG2-expressing cells without MG132 treatment. Quantitative data from three independent experiments were quantified by one-way ANOVA, $^{*}P < 0.05$. (B) BAG2 stabilised the R492X PINK1 mutation. HEK293 cells were co-transfected with EGFP-N1 or EGFP-N1-BAG2 together with HA-PINK1-R492X, with or without MG132 treatment. The PINK1-R492X levels were increased in the EGFP-BAG2-expressing cells without MG132 treatment. Quantitative data from three independent experiments were quantified by one-way ANOVA, $^{*}P < 0.01$.

We also coexpressed EGFP-N1 or EGFP-BAG2 with the HA-R492X PINK1 mutation in HEK293 cells. The result showed a more markedly decreased ubiquitination in the EGFP-BAG2 group (Fig. 3).

4. Discussion

Because PINK1 is the second most common virulence gene besides Parkin, it is important to know about the upstream and downstream proteins in the pathogenesis pathway of PD because there is still little known. Here, with immunofluorescence, co-immunoprecipitation and pull down experiments, we present a direct physical interaction between BAG2 and PINK1. When BAG2 was overexpressed in cells, the degradation of PINK1 decreased, and in the pathogenesis-related R492X PINK1 mutation, this degradation decreased more markedly. Therefore, we proved BAG2, an upstream protein of PINK1, has important roles in regulating PINK1

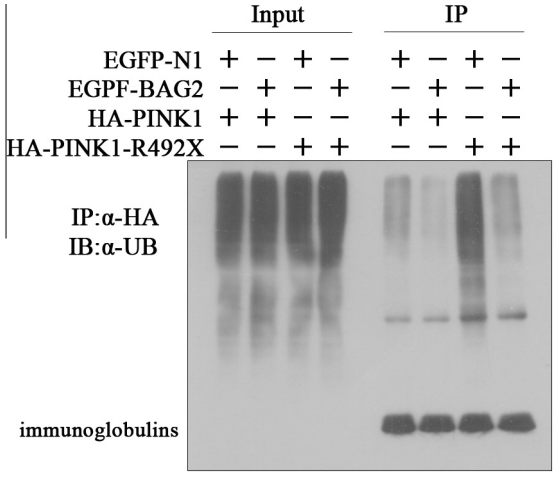

Fig. 3. BAG2 decreases the ubiquitination of PINK1 and the R492X PINK1 mutation. HEK293 cells were co-transfected with EGFP-N1 or EGFP-N1-BAG2 together with HA-PINK1 or HA-PINK1-R492X, followed by treatment with the proteasome inhibitor MG132 (5 mM) for 24 h. The lysates were subjected to IP with an anti-HA antibody, then the IP and the input lysates were analysed by immunoblotting with anti-UB antibodies.

stability and more markedly in regulating the pathogenesis-related R492X PINK1 mutation stability.

Interestingly, BAG2 binds with the R492X PINK1 mutation more tightly than with PINK1. BAG2 also decreases the ubiquitination degradation of the R492X PINK1 mutation more obviously, which means that BAG2 has a more important influence on PD patients than normal people. An explanation for this may be that the R492X PINK1 mutation induces cellular mitochondrial dysfunction [23] and at the same time, the R492X PINK1 mutation aggravates the cellular ubiquitin proteasome pathway burden, but the intracellular ubiquitin conjugating substrate requires ATP to provide energy. Therefore, cells overexpressing the R492X PINK1 mutation are more vulnerable and easier to imbalance than cells overexpressing PINK1.

Important roles for BAG family proteins in neurodegenerative diseases continue to be discovered. BAG1, the first discovered BAG family protein [24], has been reported to regulate cell growth [25–28], to regulate the proteasomal degradation of tau protein [29,30] and to modulate huntingtin toxicity and degradation that is dependent on an intact BAG domain [31–33]. BAG5 inhibits parkin [34,35] and regulates the ubiquitinylation of α-synuclein [36]. Moreover, BAG2 directs tau to a ubiquitin-independent pathway [20]. We show that BAG2 stabilises PINK1 through the ubiquitin proteasome pathway. Thus, our data directly correlate BAG2 to PD-related neurodegeneration, strengthening the link between the BAG proteins and PD-related neurodegeneration.

In Drosophila, a PINK1 deficiency was shown to affect mitochondrial function and morphology [10,37,38]. In cells, the downregulation of PINK1 results in abnormal mitochondrial morphology and altered membrane potential. This downregulation also elicits oxidative stress, but up-regulated wild-type PINK1 can rescue these abnormalities [39,40]. According to our report, BAG2 stabilises PINK1 through a direct interaction. Therefore, BAG2 plays a protective effect in cells and we speculate that BAG2 also has protective effect in PD patients.

In summary, our data indicated that BAG2 plays a critical role in the ubiquitin proteasome pathway of PINK1 degradation, especially in this pathway for the degradation of the R492X PINK1 mutation. This research will lead to an understanding of the pathogenesis of PD or even novel therapeutic approaches.

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