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Research Article

Parkin Co-Regulated Gene is involved in aggresome formation and autophagy in response to proteasomal impairment

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ABSTRACT

PArkin Co-Regulated Gene is a gene that shares a bidirectional promoter with the Parkinson's disease associated gene *parkin*. The encoded protein (PACRG) is found in Lewy bodies and glial cytoplasmic inclusions, the pathological hallmarks of parkinsonian disorders. To investigate the function and regulation of PACRG, cells were treated with the proteasomal inhibitor, MG-132. As previously reported with parkin, inhibition of the proteasome resulted in the formation of aggresomes that contained endogenous PACRG. Increased levels of exogenous PACRG resulted in an increase in aggresome formation, and conferred significant resistance to aggresome disruption and cell death mediated by microtubule depolymerisation. In contrast, shRNA mediated knockdown of *PACRG* significantly reduced aggresome numbers. Elevated levels of PACRG also resulted in increased autophagy, as demonstrated by biochemical and quantitative analysis of autophagic vesicles, whereas lowered levels of PACRG resulted in reduced autophagy. These results suggest a role for PACRG in aggresome formation and establish a further link between the UPS and autophagy.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder affecting greater than 1% of individuals over the age of 65 [1]. The disease is characterised by a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) with the presence of intracytoplasmic eosinophilic inclusions termed as Lewy bodies (LBs) in many of the surviving neurons [2]. The current understanding of PD pathogenesis is incomplete, however the ubiquitin-proteasome system (UPS) has been implicated

Abbreviations: 3-MA, 3-methyladenine; AV, autophagic vesicle; BSA, bovine serum albumin; ER, endoplasmic reticulum; FBS, foetal bovine serum; LB, Lewy body; LC3, light chain 3; MT, microtubule; MTOC, microtubule organising centre; PACRG, Parkin Co-Regulated Gene; PD, Parkinson's disease; PBS, phosphate buffered saline; SNpc, substantia nigra pars compacta; TEM, transmission electron microscopy; TBS, tris buffered saline; UPS, ubiquitin proteasomal system; UPR, unfolded protein response

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by a number of studies suggesting that the neurodegenerative process is due in part to the accumulation of misfolded or toxic proteins [3]. The UPS is a major pathway for the turnover of intracellular proteins. In addition to the degradation of misfolded/malfunctioning proteins, the UPS plays an important role in many basic cellular processes, including regulation of cell cycle and division, differentiation and development [4].

Cell models are a useful tool to investigate the potential involvement of the UPS and misfolded proteins in PD pathogenesis. Previous studies have demonstrated that when the capacity of a cell to degrade misfolded proteins is exceeded perinuclear inclusions termed as aggresomes may be formed [5]. This can occur in response to cellular stresses, such as proteasome inhibition or heat shock, or alternatively when the steady-state levels of certain proteins including the PD associated proteins parkin [6], synphilin [7], UCH-L1 [8], α-synuclein [9] or p38 [10] are elevated. Aggresomes develop at the microtubule organising centre (MTOC) from smaller aggregates that have been retrogradely transported by microtubules to the MTOC [11]. Relatively little is known about the mechanisms by which aggresomes are formed, or which proteins are important in this process. Similarly, the cellular role of aggresomes is unclear. It has been suggested that they represent a protective response to cell stress by sequestering misfolded or toxic proteins for subsequent removal by proteasome and/or autophagy mediated mechanisms (reviewed in [12]).

The novel gene, Parkin Co-Regulated Gene (*PACRG*) is oriented head to head with *parkin* and the two genes share a bidirectional promoter [13]. The protein has previously been implicated in the unfolded protein stress response, cilia/flagella function and susceptibility to leprosy and typhoid fever [14–18]. However, the significance of the coregulation of *PACRG* and *parkin* and the functional role of PACRG is not well understood. We hypothesise that both PACRG and parkin function in a common molecular pathway. Parkin has previously been shown to stabilise microtubules and potentially mediate the formation of aggresomes [19–21]. There is some evidence that PACRG is a component of aggresomes and we have recently demonstrated that PACRG is tightly regulated by the UPS and is upregulated in parkinsonian syndromes [14,22]. Notably, PACRG is a component of the pathological hallmarks of these diseases including Lewy bodies, glial cytoplasmic inclusions and neurofibrillary tangles. In this study, we have investigated the role of PACRG in the formation of aggresomes and the induction of autophagy induced by proteasomal inhibition.

Materials and methods

Plasmids, antibodies and reagents

Plasmids expressing PACRG, V5His-tagged PACRG or GFPμ have been described previously [22,23]. The following primary antibodies were used: monoclonal anti-V5 (1:1000, R960-25, Invitrogen), monoclonal anti-vimentin (1:1000, V2258, Sigma), monoclonal anti-α-tubulin (1:1000, T5168, Sigma), monoclonal anti-γ-tubulin (1:1000, T6557, Sigma), anti-GFP (1:2500, MAB3580, Chemicon), monoclonal anti-Hsp70 (1:1000, SPA-810, Stressgen), polyclonal anti-LC3 (1:2000, PM036, MBL), monoclonal anti-p62 (1:2000, 610832, BD Transduction Laboratories) and monoclonal anti-β-actin (1:5000, A5441, Sigma). A human specific polyclonal antibody directed against the N-terminal sequence of PACRG (MVAEKETLSLNKC, MC1250) was generated as previously described [13]. The specificity of this antibody was confirmed by western blot analysis (concentration of 0.5 μg/mL) of recombinant PACRG protein (Supplementary Fig. 1A) and by immunohistochemistry (concentration of 1 μg/mL) (Supplementary Fig. 1B) and revealed similar PACRG staining and localisation as the previously described anti-PACRG antibody MC1290 [24]. Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 (1:1000, A11034, Molecular Probes) and goat anti-mouse Alexa Fluor 594 (1:1000, A11032, Molecular Probes). Stock solutions of the following chemicals were prepared and stored at −20 °C: MG-132 (50 mM in DMSO, Merck), lactacystin (50 mM in DMSO, Sigma), 3-methyladenine (62 mM in H_2O, Sigma), rapamycin (2 mM in DMSO, Merck) and nocodazole (1 mg/mL in DMSO, Sigma).

Cell culture and treatments

Human embryonic kidney cells (HEK-293T) and human neuroblastoma BE(2)M17 (M17) cells were obtained from the American Type Culture Collection (ATCC) and were cultured in Opti-MEM (Invitrogen) supplemented with 10% (v/v) FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). To generate PACRG overexpressing cell lines, HEK-293T or M17 cells were transfected with pcDNA3.1-PACRG-V5His or pcDNA3.1-PACRG, respectively using Fugene HD (Roche). Clonal cell lines stably overexpressing PACRG were isolated by limiting dilution and maintained in 400 μg/mL geneticin. Cells were plated 24 h prior to treatments in six-well culture plates at 2×10^5 cells per well for immunohistochemistry or in 24-well plates at 6×10^4 cells per well for cell viability assays and maintained at 37 °C/5% CO_2. Cells were treated with the indicated doses or the carrier solvent control DMSO for 16 h unless otherwise stated. In assessing the role of autophagy in clearing aggresomes, the method of Fortun [25] was used. Cells were initially treated for 16 h with 10 μM lactacystin, washed twice in PBS, before allowing cells to recover in the indicated conditions for 24 h and aggresomes analysed as below.

For *PACRG* knockdown experiments, cells in six-well plates were transiently transfected for 48 h using FugeneHD (Roche) with 1 μg of a HuSH shRNA plasmid containing a *PACRG* specific shRNA cassette with a GFP tag (Origene). Cells were then treated for 16 h with MG-132 (or vehicle) and harvested for western blot analysis. Two independent constructs were used to knockdown *PACRG* expression and a verified plasmid without an shRNA cassette insert (TR20003) was used as a negative control.

Immunofluorescence

Cells were seeded onto sterile gelatin-coated coverslips in six-well culture plates and treated as described. Cells were fixed for 15 min at room temperature in 4% (w/v) paraformaldehyde, washed in phosphate-buffered saline (PBS) before being permeabilised with 0.2% (v/v) Triton/PBS for 20 min at room temperature. Blocking was performed in 10% (v/v) normal goat serum with 5% (w/v) bovine serum albumin in PBS for 1 h at room temperature. Primary antibodies were diluted in PBS with 1% (w/v) BSA, applied and allowed to incubate for 20 h at 4 °C. Cells were then washed twice in PBS for 5 min and incubated with secondary antibodies for 1 h at room temperature. Cells were