Biochemical and Biophysical Research Communications 391 (2010) 800–805

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Naturally- and experimentally-designed restorations of the *Parkin* gene deficit in autosomal recessive juvenile parkinsonism

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ARTICLE INFO

Article history:
Received 20 November 2009
Available online 27 November 2009

Keywords:
Parkinsonism
Parkin
Cyclin E
Antisense oligonucleotide
Exon skipping

ABSTRACT

Intranuclear events due to mutations in the *Parkin* gene remain elusive in autosomal recessive juvenile parkinsonism (ARJP). We identified a mutant PARKIN protein in fibroblast cultures from a pair of siblings with ARJP who were homozygous for the exon 4-deleted *Parkin* gene. Disease was mild in one patient and debilitating in the other. The detected mutant, encoded by a transcript lacking exon 3 as well as exon 4, is an in-frame deletion that removes 121 aa, resulting in a 344-aa protein (PaDel3,4). Cell culture and transfection studies revealed negative correlations between expression levels of PaDel3,4 and those of cell cycle proteins, including cyclin E, CDK2, ppRb, and E2F-1, and demonstrated that GFP-PaDel3,4 entered nucleus and ubiquitinated cyclin E as a part of $SCF^{hSel-10}$ ligase complex in the patient cells. In addition, nuclear localization signal-tagged PaDel3,4 expressed in the transfected patient cells most effectively ubiquitinated cyclin E and reduced DNA damage, protecting cells from oxidative stress. Antisense-oligonucleotide treatment promoted skipping of exon 3 and thus generated PaDel3,4, increasing cell survival. Collectively, we propose that naturally- and experimentally-induced exon skipping at least partly restores the mutant *Parkin* gene deficit, providing a molecular basis for the development of therapeutic exon skipping

Introduction

Autosomal recessive juvenile parkinsonism (ARJP) is caused by mutations in the gene encoding the E3 ubiquitin ligase PARKIN [1]. Mutated PARKIN fails to label substrates with ubiquitin chains for proteasomal degradation, leading to their toxic accumulation. This theory is based primarily on the results of molecular studies focusing on processes in cytoplasm, a major territory for PARKIN [2,3], while intranuclear events due to *Parkin* mutations have received little attention. The critical role played by oxidative stress in neurodegeneration in ARJP was initially suggested by the finding that patients with this disease had iron accumulation in the substantial nigra (SN), an important indicator of oxidative damage [4]. A growing body of evidence has further indicated that oxidative stress promotes cell cycle activation, leading to neuron death [5–7]. Consistent with this notion, cell cycle proteins are detectable in the SN of patients with Parkinson's disease (PD) [8,9]. Cyclin E deserves particular attention because it accumulates in ARJP brain, and most importantly, is a putative substrate of PARKIN [10]. Interest has focused on possible interactions between intranuclear mutant PARKIN and cyclin E to better understand the pathomechanisms of ARJP. New therapies are clearly needed for this disease. Conventional dopamine replacement therapy alleviates symptoms only transiently; its usefulness wanes over time and unacceptable adverse effects often develop, particularly problematic in ARJP [1].

In this study, we assessed the influence of oxidative stress on cell death by analyzing interactions between mutant PARKIN and cyclin E in cultured fibroblasts from a pair of siblings with ARJP. The patients provided an experimental cell model to study intranuclear mechanisms by which ARJP cells protest against oxidative stress. Our results may provide a molecular basis for the development of antisense-oligonucleotide (AON)-mediated therapeutic interventions in ARJP.

Materials and methods

Patients and analyses of the Parkin gene in fibroblast cultures. A 75-year-old Japanese man had a 48-year history of parkinsonism. Despite undergoing a thalamotomy when he was 37 years old, current symptoms were debilitating, estimated as 90/260 on the Movement Disorder Society-sponsored revision of the United Parkinson's Disease Rating Scale (MDS-UPDRS) [11]. His 72-year-old

Abbreviations: ARJP, autosomal recessive juvenile parkinsonism; AON, antisense oligonucleotide; BSO, buthionine-(*S,R*)-sulfoximine; GSH, glutathione; LMB, leptomycin B; NLS, nuclear localization signal; NES, nuclear export signal; 8-oxoG, 8-hydroxyl-deoxyguanine; SCF, Skp1/cullin/F-box.

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.11.141

sister had an 8-year history of slow gait with a MDS-UPDRS score of 12/260. Both patients provided written informed consent to this study. Fibroblasts were obtained by skin biopsy from the patients and age-matched controls and were cultured [12]. The sequence of the *Parkin* gene was analyzed, and deletion breakpoints were identified as described previously [13]. The primary fibroblast culture from the sibling with mild parkinsonism was termed mPDfib, and that from the sibling with severely debilitating parkinsonism was termed sPDfib. The materials used in this study and the methods for quantitative analyses of gene and mRNA, cell survival assay under oxidative stress (buthionine-(*S*,*R*)-sulfoximine [BSO] treatment), immunoblot, and immunocytochemical studies are described in detail in Supplementary materials and methods.

Vector construction and transfection. cDNAs encoding wild type (WT)-PARKIN and all mutants (PARKIN^{T415N}, PaDel3,4, and PaDel3,4^{T415N}) were synthesized by normal and mutagenic PCR and were inserted into pEGFP-C1 (Clontech Laboratory, Inc., Mountain View, CA) and pCold (Takara Bio, Inc., Shiga, Japan). Nuclear localization signal (NLS) and REV nuclear export signal (NES) were used for specific subcellular targeting [12,14]. Cyclin E cDNA was inserted into pGEX-6P-2 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Hemagglutinin (HA)–ubiquitin and *myc*-hSel-10 cDNAs were provided by Drs. Tanaka and Vogelstein, respectively. Cells were transfected with plasmid DNAs using Lipofectamine 2000.

Ubiquitination assay in vivo and in vitro. For *in vivo* ubiquitination analysis of endogenous cyclin E, fibroblasts (4×10^4 cells/well) were transfected with 2 μg of HA–ubiquitin and 2 μg of various *Parkin* plasmids or AON. Transfected-cell lysates were immunoprecipitated with anti-cyclin E and analyzed by immunoblotting with anti-HA. In reciprocal experiments, lysates were immunoprecipitated with anti-HA and immunoblotted with anti-cyclin E. For *in vitro* ubiquitination assay, 50 μl of a mixture containing 10 μg of ubiquitin, 100 μg of E1, 200 μg of UbcH7, GST-column (Amersham) purified recombinant cyclin E, and Ni-column (Qiagen) purified recombinant PARKIN or PaDel3,4 was incubated for 2 h at 37 °C. Immunoprecipitated *myc*-tagged hSel-10 (from lysates of sPDfib transfected with *myc*-hSel-10) was also added. An equal volume of 2× SDS sample buffer was added to terminate the reaction. Proteins were resolved on 5–20% SDS–PAGE gels and analyzed by Western blotting technique.

Antisense treatment. A target sequence for AON (PASex3: 5′-CAUUUCUUGACCUUUUCUCC-3′) was set on a potential splicing enhancer sequence (a polypurine region) in exon 3 of the *Parkin* gene (cDNA nucleotides 221–240; A of the first ATG counted as 1) [15]. A scramble oligonucleotide (SC1: 5′-CCUUUCUUCGUCCUUCUAAU-3′) was used as control. The oligonucleotides contained a 5′-fluorescein group, a full-length phosphorothioate backbone, and 2′-*O*-methyl modified ribose molecules (Biologica, Inc., Nagoya, Japan). Fibroblasts were transfected separately with these oligonucleotides using Lipofectamin 2000 and subjected to protein analyses.

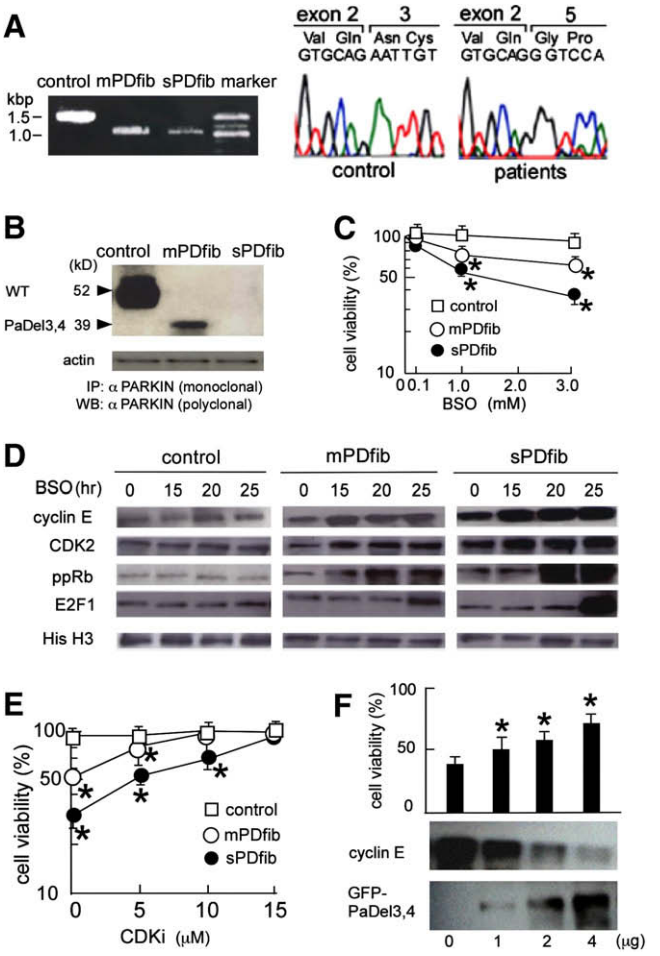

Fig. 1. PaDel3,4 identification and cell cycle proteins in ARJP fibroblasts. (A) RT-PCR and sequence analyses showed WT-*Parkin* transcript in control cells, and a short PaDel3,4 transcript lacking exons 3 and 4 in patient cells (mPDfib and sPDfib). (B) Identification of endogenous WT-PARKIN (52 kDa) in control, and PaDel3,4 (39 kDa) in mPDfib, but not in sPDfib. Immunoprecipitated (IP) cell lysates with anti-PARKIN monoclonal antibody were analyzed by Western blotting (WB) using anti-PARKIN polyclonal antibody. (C) Survival of control and patient cells under oxidative conditions. Fibroblasts were treated with 0–3 mM BSO for 48 h and subjected to cell viability assay. $^{*}P < 0.01$, Mann–Whitney *U* test as compared with control cells. (D) Time-dependent increases of cell cycle proteins in nuclear lysates from 3 mM BSO-treated cells. His H3 represents a nuclear fraction marker. (E) CVT-313 (CDKi) dose-dependently protected patient cells from BSO. (F) Exogenous PaDel3,4 dose-dependently protected sPDfib from BSO, accompanied by a reduction in endogenous cyclin E. sPDfib was transfected with 0–4 μg of GFP-PaDel3,4 vector and incubated in 3 mM BSO-containing medium for 25 h (WB) and 48 h (cell survival assay). The bar represents the means ± SD from five independent experiments. $^{*}P < 0.01$, Mann–Whitney *U* test as compared with mock-transfection sample.

Results

Frame-restoring exon skipping results in PaDel3,4 in ARJP fibroblasts

The ARJP siblings were homozygous for the mutant *Parkin* gene with exon 4-deletion, and the 11 remaining exons had normal sequences. A breakpoint was located 25,481 base pairs (bp) upstream and 56,137 bp downstream of exon 4 in both alleles of the patients' *Parkin* genes. With the primers flanking exons 1–12 cDNA, RT-PCR analyses of normal controls amplified a transcript of an expected size (1521 bp), whereas analyses of the patients' genes produced a single short transcript (1158 bp) lacking exon 3 as well as exon 4. This was an in-frame deletion that removed 121 amino acids (aa) (58–178 aa), resulting in the 344-aa mutant PARKIN, PaDel3,4 (Fig. 1A). PaDel3,4 may represent 75% of the ubiquitin-like domain and an entire RING box, presumably retaining E3 ubiquitin ligase activity [1–3]. Real-time quantitative RT-PCR revealed that the PaDel3,4 mRNA level in mPDfib was significantly (about 4 times) higher than that in sPDfib (Supplementary Fig. 1). Consistent with the higher level of mRNA, the corresponding protein (39 kDa) was readily detectable in mPDfib by immunoprecipitation-Western blotting technique. Highly sensitive, short-range (exons 3–5) but not long-range RT-PCR (exons 1–12) detected the transcript lacking exon 4 only in the patients at a very low level on quantitative analysis (not shown), possibly because this truncated transcript carries a premature termination codon, which may be subjected to "nonsense-mediated mRNA decay". Despite the low level, identification of this transcript may be important, since it might support expression of the transcript (or pre-mRNA) retaining exon 3 and may exclude inversion of this exon. It was particularly

important that sPDfib (from the patient with severe ARJP) had no PARKIN-relevant proteins and thus represented a spontaneously occurring model of PARKIN-deficient cells (Fig. 1B).

Susceptibility of ARJP fibroblasts to oxidative stress is related to PaDel3,4

To test whether the *Parkin* gene mutation sensitizes ARJP fibroblasts to oxidative stress, we added BSO to the culture medium. BSO is a highly selective inhibitor of γ-glutamylcysteine synthetase, which effectively arrests glutathione (GSH) synthesis when added to culture medium. In this model of oxidative stress, the endogenous formation of reactive oxygen species is largely unopposed, consequently resulting in oxidative cell damage [12]. Cell survival assay and Western blot analysis of nuclear lysates revealed that the patient cells had increased sensitivity to BSO and higher expression levels of cyclin E, CDK2, ppRb, and E2F-1, more prominently in sPDfib than in mPDfib, leading to cell death (Fig. 1C and D). CDKi suppressed BSO-induced ARJP cell death (Fig. 1E). Furthermore, transfection of sPDfib with PaDel3,4 vector dose-dependently restored tolerability to BSO, accompanied by a reduction in endogenous cyclin E (Fig. 1F). Collectively, we suggest that PaDel3,4 modifies susceptibility of patient cells to oxidative stress by suppressing oxidative-stress-induced accumulation of cell cycle proteins.

PaDel3,4 ubiquitinates cyclin E in vivo and in vitro

We examined whether endogenous PaDel3,4 could ubiquitinate cyclin E in ARJP fibroblasts cultured in BSO-free medium. *In vivo* ubiquitination assay followed by Western blotting revealed that control and mPDfib showed significant anti-HA and anti-cyclin E immunoreactivity in the form of smear, a characteristic of polyubiquitinated protein, suggesting that PaDel3,4 ubiquitinated cyclin E. This ubiquitination was confirmed by reciprocal experiments. As expected, sPDfib displayed cyclin E accumulation but no smear, confirming that sPDfib represents a PARKIN-deficient cell model (Fig. 2A). PARKIN functions as a part of SCF (Skp1/cullin/F-box) ligase complexes, and hSel-10 (an F-box/WD protein) targets the ubiquitin ligase activity to cyclin E [10]. Consistent with this notion, immunoprecipitation of cell lysates with anti-PARKIN antibody coimmunoprecipitated both hSel-10 and cyclin E in normal cells and mPDfib (Fig. 2B, left). To demonstrate that hSel-10 is essential for cyclin E ubiquitination by PaDel3,4, we performed an *in vitro* ubiquitination assay. Reaction mixtures containing recombinant forms of WT-PARKIN or PaDel3,4, cyclin E, ubiquitin, E1, and E2 (UbcH7) were incubated in the presence or absence of immunopurified *myc*-hSel-10. Western blotting with anti-cyclin E and anti-ubiquitin antibodies showed high molecular weight smear in control and mPDfib only in the presence of hSel-10 (Fig. 2B, right). All together, our results suggested that PaDel3,4 as well as WT-PARKIN function as components for $SCF^{hSel\text{-}10}$ complex in cyclin E ubiquitination. The deduced amino acid sequence indicated that PaDel3,4 lost 121 aa (58–178 aa) corresponding to part of the membrane-binding domain at the 77–178 aa moiety, no longer localized to cytoplasm [16]. In fact, fluorescent imaging and immunostaining showed that PaDel3,4 predominantly localized to the nucleus, whereas hSel-10 resided throughout the cytoplasm and nucleus, supporting intranuclear interactions between the two proteins (Fig. 2C).

Nuclear localized PaDel3,4 ubiquitinates cyclin E and reduces DNA damage, contributing to cell survival

To directly verify that PaDel3,4 functions as an E3 ubiquitin ligase within nucleus of ARJP fibroblasts, we constructed GFP-PaDel3,4 expression vector carrying NLS. The specific signal mstNLS, corresponding to 239–276 aa of X-ray repair cross-complementing

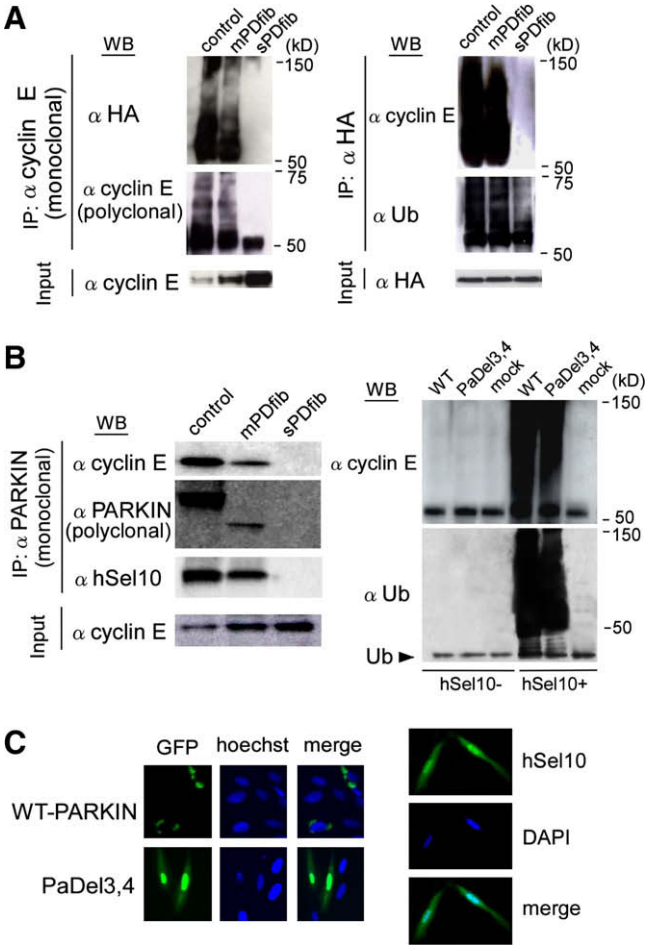

Fig. 2. WT-PARKIN and PaDel3,4 ubiquitinated cyclin E. (A) *In vivo* ubiquitination of cyclin E in control and ARJP fibroblasts transfected with HA–ubiquitin expression vector. Immunoprecipitated (IP) cell lysates with anti-cyclin E were analyzed by Western blotting (WB) using anti-HA and cyclin E antibodies (left), as confirmed by immunoprecipitation of the same cell lysates with anti-HA antibody, followed by blotting with anti-cyclin E and anti-ubiquitin antibodies (right). Note that sPDfib can be a PARKIN-deficient cell model. (B) Cyclin E is a candidate substrate of the WT-PARKIN- and PaDel3,4-$SCF^{hSel\text{-}10}$ complex. On immunoprecipitation of cell lysates with anti-PARKIN monoclonal antibody, both cyclin E and hSel-10 were contained in control fibroblasts and mPDfib, but not in sPDfib (left). *In vitro* ubiquitination of cyclin E by WT-PARKIN and PaDel3,4 only in the presence of hSel-10 (right). (C) Subcellular localization of GFP-WT-PARKIN and GFP-PaDel3,4 in PARKIN-deficient sPDfib (left). *Myc*-tagged hSel-10 was localized in the nucleus and cytoplasm (right).

group 1 (XRCC1), comes from the extension of the classical NLS for the *karyopherin*-mediated nuclear import of cargo proteins [12,14]. With this vector, we concentrated PaDel3,4 within the nucleus, as evidenced by colocalization of GFP signal with Hoechst33258-stained nuclear DNA in sPDfib (Fig. 3A). The nuclear lysates prepared from sPDfib overexpressed with NLS-tagged PaDel3,4 along with HA–ubiquitin were immunoprecipitated with monoclonal anti-cyclin E antibody. Endogenous cyclin E is ubiquitinated by PaDel3,4, as shown by the anti-HA immunoreactivity in the form of smear, suggesting that PaDel3,4 ubiquitinated cyclin E within nucleus, as confirmed by reciprocal experiments (Fig. 3B, lane 4). These results were consistent with those of parallel experiments using mstNLS-tagged $PaDel3,4^{T415N}$ vector. T415N located within RING box abolished E3 ligase activity (Fig. 3B, lane 6). Cell survival assay showed that mstNLS-tagged PaDel3,4 protected sPDfib from BSO stress, accompanied by reduced DNA damage as demonstrated by staining with 8-oxoG in the 4′,6-diamino-2-phenylindole-stained nucleus. mstNLS-$PaDel3,4^{T415N}$ vector abolished the

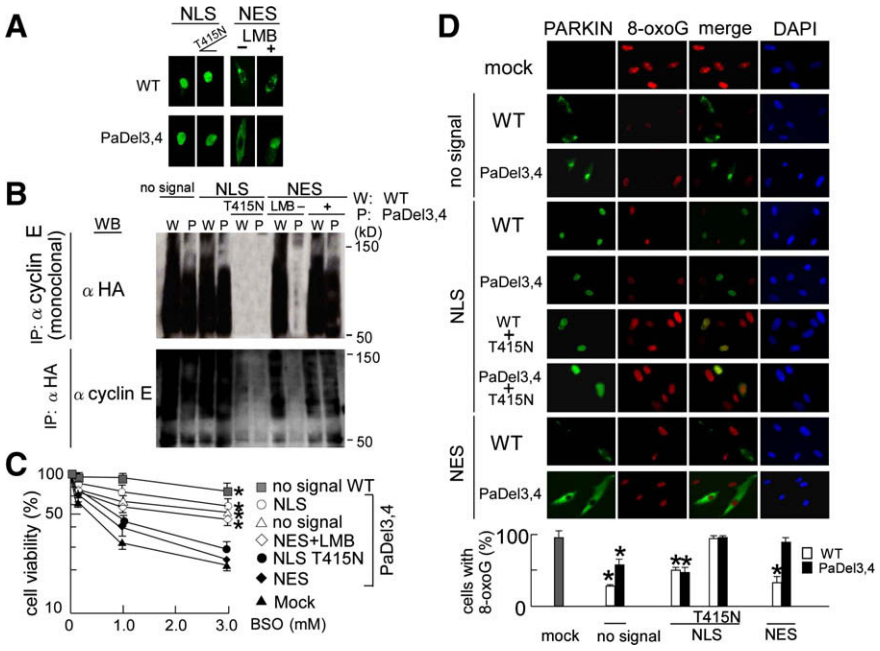

Fig. 3. Nuclear localized PaDel3,4 ubiquitinated cyclin E and reduced DNA damage, improving sPDfib survival. (A) mstNLS-GFP-tagged WT-PARKIN, WT-PARKIN^{T415N}, PaDel3,4, and PaDel3,4^{T415N} were localized in the nucleus. NES-GFP-tagged PARKIN and PaDe3,4 were localized in the cytoplasm, and PaDel3,4 was localized in the nucleus with leptomycin B (LMB). (B) PaDel3,4 effectively ubiquitinated cyclin E within nucleus (upper and lower panels, lane 4), whereas NLS-tagged PaDel3,4^{T415N} did not (lane 6). NES-GFP-PaDel3,4 vector reduced cyclin E ubiquitination, which was restored by leptomycin B (lanes 8 and 10). (C) sPDfib was transfected with different signal-tagged PaDel3,4 and incubated for 48 h in 0–3 mM BSO. mstNLS-PaDel3,4 markedly improved sPDfib survival, whereas mstNLS-PaDel3,4^{T415N} and NES-PaDel3,4 did not. The bar represents SD from five independent experiments. $^{*}P < 0.01$, Mann–Whitney *U* test as compared with mock-transfected cells. (D) Immunocytochemistry of sPDfib expressing GFP-WT-PARKIN or GFP-PaDel3,4 (green) tagged with mstNLS or NES. Cells were incubated for 10 h in 1.5 mM BSO medium. 8-oxoG was stained with Alexa 594 (red). Nuclei were counterstained with DAPI (blue). Nuclear import of PaDel3,4 protected cells from oxidative stress, whereas cytoplasmic targeting showed no such effects ($^{*}P < 0.05$, unpaired Student's *t* test as compared with mock-transfected cells). The bar represents the means ± SD from five independent experiments (lower panel).

protective effects (Fig. 3C and D). In similar experiments except for a use of NES-tagged PaDel3,4, cytoplasmic targeting of PaDel3,4 hardly ubiquitinated cyclin E (Fig. 3B, lane 8) and failed to protect the patient cells from oxidative stress (Fig. 3C and D). Taken together, this study uncovered the intranuclear mechanism by which patient cells were protected against oxidative stress via PaDel3,4-mediated regulation of the cell cycle and DNA damage.

AON PASex3-induced PaDel3,4 contributes to ARJP cell survival

PaDel3,4, which partially protects ARJP fibroblasts from oxidative stress, is a potential target for therapeutic interventions. We synthesized the AON complementary to a 20-nucleotide sequence of exonic splicing enhancer on exon 3 of the *Parkin* gene. This antisense protein, termed PASex3, inhibited exon 3 inclusion in an out-of-frame transcript lacking exon 4, leading to an in-frame transcript in sPDfib. PASex3 considerably increased PaDel3,4 protein in the patient cells (Fig. 4A). Lysates from PASex3-treated sPDfib were immunoprecipitated with anti-cyclin E monoclonal antibody and analyzed by Western blotting using polyclonal antibodies to HA and cyclin E. The loss of cyclin E and simultaneous gain of high molecular anti-HA reactive smear indicated that these species represent ubiquitinated forms of cyclin E in the patient cells. This ubiquitination was further confirmed by reciprocal experiments (Fig. 4B). PASex3 promoted increased cell viability against BSO-induced oxidative stress (Fig. 4C).

Discussion

In this study, we identified and characterized mutant PARKIN, termed PaDel3,4, which contributed to delaying the death of ARJP fibroblasts exposed to oxidative stress. Cell cultures can thus be a robust disease model for the development of therapeutic exon skipping procedures.

Spontaneous exon skipping leads to restoration of the tolerability of ARJP fibroblasts to oxidative stress

Previously identified pathogenic mutations of the *Parkin* gene do not systematically abrogate E3 ubiquitin ligase activity, suggesting that various molecular mechanisms underlie neuronal

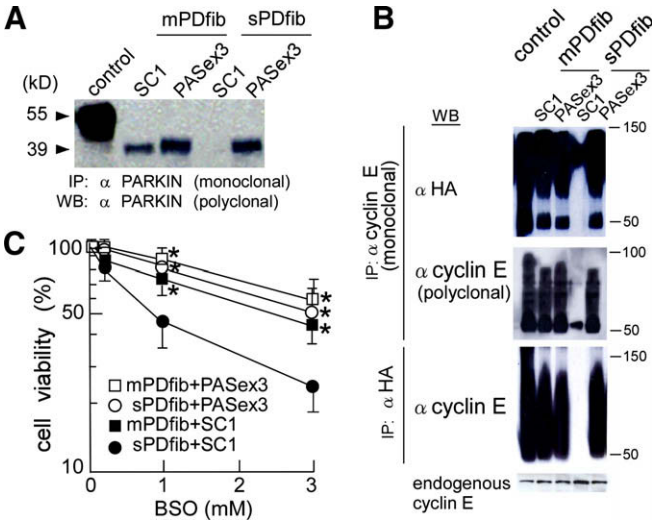

Fig. 4. AON PASex3 increases endogenous PaDel3,4, protecting patient cells from oxidative stress. (A) PASex3 increased PaDel3,4 in the patient cells. Control scrambled oligonucleotide (SC1) failed to do so. (B) Polyubiquitination of cyclin E in the patient cells treated with PASex3. (C) PASex3 increased viability of the patient cells during 48 h incubation with BSO. The bar represents the SD from five independent experiments. $^{*}P < 0.01$, Mann–Whitney *U* test as compared with SC1-transfected sPDfib.

degeneration in ARJP [17–20]. Cell survival studies and time course analyses of cell cycle proteins have shown that susceptibility of patient cells to oxidative stress partially depends on endogenous PaDel3,4. We further assumed that PaDel3,4 ubiquitinated cyclin E for degradation, thereby activating G1/S checkpoints to delay cell cycle progression and allow repair of DNA damage. This hypothesis is supported by our finding that PaDel3,4 experimentally concentrated in the nucleus most effectively ubiquitinated cyclin E and reduced DNA damage, contributing to cell survival under oxidative conditions. In contrast, cytoplasmic targeting of PaDel3,4 had virtually no beneficial effect on ARJP cell survival. Overexpression of Del3–4 (the same mutant as PaDel3,4) has been reported to weakly, but significantly increase survival of transfected SH-5YSY cells after UV-induced DNA damage [21]. Available evidence suggests that PaDel3,4 promotes DNA repair and protects cells against oxidative stress and indicates that DNA damage is an important pathogenic mechanism in ARJP.

PaDel3,4 is translated from aberrantly spliced transcripts in which frame-restoring exon skipping occurred during pre-mRNA splicing. We suggest that splicing efficacy responsible for PaDel3,4 production may account for the differences in the clinical phenotypes of the two sibling patients. This proposal is supported by an analogous system in Duchene's muscular dystrophy (DMD); the frame-shifting mutations that abrogate dystrophin synthesis cause severely debilitating DMD, whereas in-frame mutations in the same gene lead to milder Becker's muscular dystrophy [22]. Taken together, our results may provide a basis for potential therapeutic strategies based on exon skipping.

Experimental exon skipping restores the tolerability of ARJP fibroblasts to oxidative stress

Antisense PASex3 treatment of ARJP fibroblasts successfully promoted skipping of exon 3 to correct a disrupted reading-frame of the mutant *Parkin*. Thus, the produced PaDel3,4 significantly increased E3 ubiquitin ligase activity to save patient cells exposed to oxidative stress. Small increases in normal (near normal) mRNA can result in significant phenotypic improvement, and intramuscular injections of specific AON successfully induce dystrophin expression in patients with DMD [23–25]. Collectively, our results suggest that genetic-based therapy for ARJP can be developed on the basis of AON PASex3-mediated exon skipping.

The extent to which fibroblasts reflect neurons in the patient brain remains unknown. Increasing evidence supports the notion that oxidative stress and cell cycle processes may be intertwined at the molecular level in the brain of patients with ARJP. A previous postmortem examination has demonstrated abnormal accumulation of iron in SN of the patients, implying involvement of oxidative stress in the pathomechanism of ARJP [4]. In addition, cyclin E accumulates in patients' SN [10], and neurons exhibit aberrant cell cycle activation in neurodegenerative disorders [5–7]. Here we clearly showed the association between oxidative stress and the cell cycle in the present experiment; BSO-treated fibroblasts up-regulated cell cycle proteins, more prominently in patients with ARJP than in controls, leading to cell death. Available evidence thus strongly suggests that fibroblasts reflect neurons in the brain of patients with ARJP. Another important puzzle is the mechanism for choosing between out-of-frame and in-frame splicing. Environmental elements are unlikely to be involved, because all tested cells in our study were exposed to identical culture conditions. Identical breakpoints localized at the deletion hot spot in the *Parkin* genes from the two sibling patients provided no clues as to other factors potentially related to the type of splicing [26]. This issue must await further extensive investigations.

In summary, our study has uncovered the nuclear pathway by which the PARKIN mutant PaDel3,4 ubiquitinates cyclin E and may thus control the cell cycle, contributing to cell survival. Our observations may provide a molecular basis for the development of an antisense-mediated therapeutic procedure that would be specific, rational, and genetically based.

Acknowledgments

The authors thank Drs. Hattori (Juntendo University, Tokyo, Japan), Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD), and Tanaka (The Tokyo Metropolitan Institute of Medical Science, Tokyo) for the gifts of plasmid vectors. This study was partly supported by Health and Labour Sciences Research Grants (Research in Intractable Diseases) and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Drs. Hirano and Ueno).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.141.

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