Alterations of DJ-1 protein levels in Parkinson's disease brains

Parkin enhances the steady-state levels of DJ-1 primarily in the detergent-insoluble fraction in cultured cells, and this may relate to increased protein stability. To explore whether this finding has potential pathophysiological relevance, we monitored the levels and detergent solubility of DJ-1 in human post-mortem cingulate cortex tissue from aged-matched normal control, Alzheimer's disease (AD) and PD/dementia with Lewy bodies (DLB) patients (Table 1; Fig. 6). Although the relative amount of DJ-1 does not change in the detergent-soluble fraction of all cases, a dramatic increase of DJ-1 levels in the detergent-insoluble fraction is observed in PD/DLB cases when compared with control or AD cases (Fig. 6A). Quantification and normalization of DJ-1 protein levels reveals a >5-fold increase of DJ-1 in the insoluble fraction of PD/DLB cases when compared with combined control and AD cases, whereas there is no apparent difference in the soluble fraction (Fig. 6A). Parkin resides only in the detergent-insoluble fraction in the aged human brain, but we fail to observe any difference in parkin levels in PD/DLB cases compared with control or AD cases (Fig. 6A). To determine whether this observation is specifically related to pathologically affected regions of PD/DLB brains, we examined DJ-1 levels in similar protein fractions derived from cerebellum tissue, a region not affected by the pathogenesis of PD/DLB. We observe no apparent differences of DJ-1 levels in the detergent-soluble or insoluble fractions of PD/DLB cases compared with control or AD cases (Fig. 6B), suggesting that increased DJ-1 levels observed in PD/DLB cingulate cortex are directly related to the pathogenesis of PD/DLB.

To determine whether the absence of parkin influences the levels of DJ-1 protein *in vivo*, we examined brain tissue from the frontal cortex of patients with parkin-linked AR-JP (due to parkin exonic deletions) and aged-matched normal controls (Fig. 6C). We find that the absence of parkin in the frontal cortex of AR-JP brains leads to a marked reduction of DJ-1 in the detergent-insoluble fraction in three out of four AR-JP cases (cases 1, 3 and 4) when compared with control cases (Fig. 6C), as revealed by densitometry. We observe no consistent change of DJ-1 levels in the detergent-soluble fraction of control and AR-JP cases, although two out of four AR-JP cases (cases 3 and 4) display noticeably reduced levels of DJ-1 when compared with all control cases (Fig. 6C). As expected, parkin is detected in the detergent-insoluble fraction of control brains, but is absent from AR-JP brains, as previously described (19,59). Consistent with the stabilizing effect of parkin on DJ-1 in cultured cells, these data might suggest that parkin is able to influence the stability of DJ-1 *in vivo*; in particular, the absence of parkin leads to a reduction of DJ-1 primarily in the detergent-insoluble fraction.

Table 1. Clinical details of human subject's tissue used for western blotting

Subjects	Diagnosis	Brain region	PMD (h)	Age (year)
Control 1	—	CING, CB	10	66
Control 2	—	CING, CB	4	74
Control 3	—	FRNTL	15	61
Control 4	—	FRNTL	6	67
Control 5	—	FRNTL	10	68
Control 6	—	FRNTL	15	49
AD 1	AD	CING, CB	14	88
AD 2	AD	CING, CB	9	80
PD/DLB 1	PD/DLB/AD	CING, CB	5	82
PD/DLB 2	PD/cortical LB	CING, CB	9	75
PD/DLB 3	PD	CING, CB	16	77
PD/DLB 4	PD/DLB/AD	CING, CB	12	84
PD/DLB 5	PD/AD	CING, CB	21	64
AR-JP 1	PD	FRNTL	15	62
AR-JP 2	PD	FRNTL	4	65
AR-JP 3	PD	FRNTL	18	52
AR-JP 4	PD	FRNTL	10	68

AD, Alzheimer's disease; PD, Parkinson's disease; DLB, dementia with Lewy bodies; LB, Lewy bodies; AR-JP, autosomal recessive juvenile-onset PD; PMD, post-mortem delay; CING, cingulate cortex; CB, cerebellum; FRNTL, frontal cortex.

DISCUSSION

The major findings of this study are that parkin selectively but differentially interacts with pathogenic mutant forms of DJ-1. Pathogenic DJ-1 mutants exhibit impairments in homo-dimer formation. Parkin fails to ubiquitinate and enhance the degradation of L166P and M26I mutant DJ-1, but instead promotes their stability. Furthermore, L166P DJ-1 may be capable of forming a protein complex with parkin, CHIP and Hsp70. Oxidative stress promotes an interaction between parkin and WT DJ-1, although this does not lead to the ubiquitination or degradation of DJ-1. Parkin-mediated alterations in DJ-1 stability may be pathogenically relevant as DJ-1 levels are dramatically increased in the detergent-insoluble fraction from sporadic PD/DLB brains, but are reduced in the insoluble fraction from parkin-linked AR-JP brains. This study links together, at multiple levels, two gene products associated with familial forms of PD that may have important implications for understanding the molecular pathogenesis of this disease.

The selective association of parkin with pathogenic mutant forms of DJ-1 suggests that these mutants might share common properties. However, the L166P mutant clearly differs from other DJ-1 mutants in that it interacts with parkin most robustly. L166P DJ-1 is a highly unstable, unfolded protein that fails to dimerize and is rapidly degraded by the 20/26S proteasome (33–35). Unfolding of the L166P mutant might conceivably promote its association with a variety of proteins or protein complexes, including components of the UPS or chaperone systems. Furthermore, L166P DJ-1 may be incorporated into higher-order protein complexes in cultured cells (35). Parkin or a parkin-associated complex does not appear to be involved in the ubiquitination or degradation of L166P DJ-1. Instead, parkin can stabilize L166P or M26I mutant DJ-1, particularly detergent-insoluble forms, through a mechanism that does not involve ubiquitination, thus arguing in favour of a protective or stabilizing role for parkin. The mechanism of parkin's unexpected stabilizing effect is unclear, but might result from delaying or reducing the normal turnover of exogenous DJ-1. Although it is possible that parkin or Hsp70 might stabilize DJ-1 by sequestration or redistribution into distinct inclusion

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

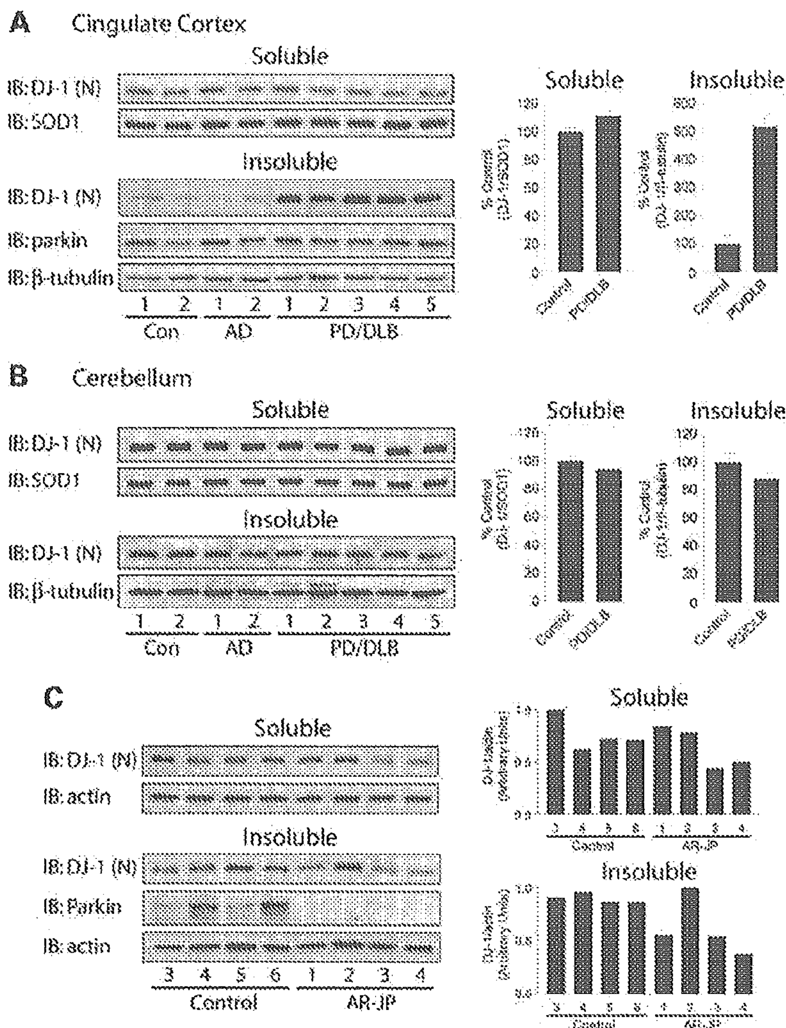

Figure 6. Alterations in DJ-1 protein levels in sporadic and parkin-linked PD brain. (**A**) Increased levels of insoluble DJ-1 in PD/dementia with Lewy bodies (DLB) brains. Equivalent detergent-soluble and -insoluble fractions extracted from cingulate cortex tissue of control (Con), AD and PD/DLB brains were analyzed by WB with anti-DJ-1 (N), anti-parkin, anti-SOD1 or anti-β-tubulin antibodies, as indicated. DJ-1 protein levels were quantified and normalized in the detergent-soluble and -insoluble fraction of control (control + AD; $n = 4$) and PD/DLB ($n = 5$) brains. Data are expressed as a percentage (%) of control levels as the ratio of DJ-1 to SOD1 (soluble fraction) or β-tubulin (insoluble fraction), and bars represent the mean ± SE. (**B**) Similar experiments were performed on detergent-soluble and -insoluble fractions extracted from cerebellum tissue of control, AD and PD/DLB brains. (**C**) Reduced levels of insoluble DJ-1 in parkin-linked AR-JP brains. Detergent-soluble and -insoluble fractions extracted from frontal cortex tissue of control and parkin-deficient AR-JP brains were analyzed by WB with anti-DJ-1 (N), anti-actin or anti-parkin antibodies, as indicated. DJ-1 protein levels were quantified and normalized in the detergent-soluble and -insoluble fraction of control and AR-JP brains. Data from individual cases are expressed in arbitrary units as the ratio of DJ-1 to actin. All experiments were replicated with similar results.

bodies or cellular organelles, this is perhaps doubtful given that this stabilizing effect is observed in both detergent-soluble and insoluble fractions. Such soluble and insoluble fractions are unlikely to simultaneously contain either inclusion bodies or organelles due to their intrinsic solubility in detergent. Thus, parkin's stabilizing effect is likely related to a more generalized effect, such that parkin binding might reduce the availability of DJ-1 for degradation. It will be of interest to determine whether parkin's stabilizing effect extends to DJ-1 harboring other pathogenic mutations, such as A104T and D149A, or the recently identified homozygous E64D mutation (60).

As L166P DJ-1 exists entirely in a non-dimeric form, a large proportion of which is monomeric (37), this might suggest that parkin selectively associates with DJ-1 monomers. Other pathogenic DJ-1 mutants demonstrate impairments in homo-dimer formation, yet still retain the capacity to form a significant population of dimers, which may lead to the availability of much smaller levels of monomeric DJ-1. This may explain the relatively weaker interaction of these DJ-1 mutants with parkin compared with the non-dimeric L166P mutant. The association of parkin with DJ-1 mutants may not be directly relevant to the pathogenesis of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PD as not all cases of DJ-1-linked PD result from missense mutations. Indeed, exonic deletions appear to be a prominent cause of DJ-1-linked PD, some of which result in a complete loss of DJ-1 protein (61). Therefore, the interaction of parkin and DJ-1 may instead link both proteins in a common molecular pathway but only under selective conditions, including pathogenic mutations or oxidative stress.

The failure of parkin to ubiquitinate or to enhance the degradation of L166P or M26I mutant DJ-1 is suggestive of an indirect interaction, perhaps as part of a larger protein complex that contains CHIP and Hsp70 together with other proteins. The function of such a protein complex is unclear. Lack of a direct parkin-mediated effect on L166P DJ-1 highlights the distinct possibility that parkin may serve subsidiary roles other than ubiquitination that might include protein stabilization or refolding. We find no evidence for polyubiquitination of L166P DJ-1 in our study, suggesting that ubiquitination does not play a major role in the proteasomal degradation of L166P DJ-1. Previous studies have similarly failed to demonstrate the formation of HMW L166P DJ-1-ubiquitin conjugates, but have instead observed the accumulation of full-length and truncated L166P DJ-1 following proteasome inhibition (33,34,36,37). The unfolded nature of L166P DJ-1 may therefore be sufficient for degradation in an ubiquitin-independent manner by the 20/26S proteasomal complex, similar to other natively unfolded proteins including α-synuclein and tau (62,63). Intriguingly, a small proportion of DJ-1 can be mono-ubiquitinated independent of parkin overexpression, raising the possibility that DJ-1 function may be regulated, in part, by non-degradative mono-ubiquitination.

The oxidative stress-induced interaction of parkin and DJ-1 failed to result in the ubiquitination or enhanced degradation of DJ-1, perhaps suggestive of an indirect interaction and/or that this association may serve an alternative biological function. Current evidence suggests that DJ-1 may function as an anti-oxidant protein and/or as a sensor of oxidative stress (44–48). Furthermore, DJ-1 may be functional under oxidative conditions because its reported chaperone and protease activities are not apparently sensitive to hydrogen peroxide treatment (37,42). As oxidative conditions do not apparently impair DJ-1, the oxidative stress-induced association of parkin and DJ-1 is unlikely the result of damaged DJ-1 protein, in contrast to the effects of pathogenic mutations, but instead may serve an alternative role. One possibility is that parkin and DJ-1 might converge in a common molecular pathway in response to oxidative stress. At present, however, the significance of the oxidative stress-induced association of parkin and DJ-1 and its relevance to the pathogenesis of PD is not understood but clearly warrants further attention.

DJ-1 levels are dramatically increased in the insoluble fraction from sporadic PD/DLB brains. The significance of this finding is not understood but might relate to increased stability of the DJ-1 protein and/or up-regulation of DJ-1 mRNA as a consequence of disease pathogenesis. Oxidative stress is one obvious candidate for enhancing DJ-1 protein levels. Parkin is primarily detergent-insoluble in the aged human brain (64). Increased levels of oxidative stress that are commonly observed in the brains of sporadic PD patients (49,50) may promote the association of parkin and DJ-1, subsequently leading to stabilization of DJ-1 exclusively in the insoluble fraction. Although this might provide an attractive putative mechanism, it is difficult to reconcile this notion with the observation that oxidative stress may actually reduce the stability of insoluble DJ-1, at least in human SH-SY5Y cells. The generally reduced levels of DJ-1 in the insoluble fraction from parkin-deficient AR-JP brains might suggest that loss of insoluble parkin may primarily destabilize DJ-1 in this fraction, consistent with the stabilizing effect of parkin in cultured cells. However, more cases need to be examined to determine whether the reduction in DJ-1 levels is a consistent feature of parkin-linked AR-JP brains. Increased DJ-1 levels in PD/DLB brains might alternatively be related to the presence of Lewy bodies or insoluble forms of α-synuclein. However, this appears unlikely given that DJ-1 fails to interact with α-synuclein and does not localize to Lewy bodies or other inclusions in sporadic PD (35,65,66). Increased levels of detergent-insoluble DJ-1 appear to be a common feature of many neurodegenerative diseases (65,66). The tendency of DJ-1 to co-localize with hallmark neuronal and glial tau inclusions in a number of neurodegenerative tauopathies likely accounts for these increased levels of insoluble DJ-1. As DJ-1 is absent from Lewy bodies in PD and DLB brains (65,66), the increased levels of insoluble DJ-1 observed in PD/DLB brains may represent a distinct pathological species with altered biochemical properties that specifically results from disease pathogenesis.

In conclusion, we report an association between the PD-linked gene products parkin and DJ-1 mediated by pathogenic DJ-1 mutations and oxidative stress. Pathogenic mutations in DJ-1 tend to impair homo-dimer formation, and parkin might be involved in stabilizing these mutants rather than targeting them for proteasomal degradation. In contrast, the oxidative stress-induced association of parkin and DJ-1 may link both proteins in a common pathway related to cellular stress. The stabilizing effect of parkin on DJ-1 appears to be one functional outcome from the association of both proteins, which may be relevant both in cultured cells and *in vivo* in PD. The association of parkin and DJ-1 at multiple levels may potentially link both proteins in a common molecular pathway, and this may have important implications for understanding the pathogenesis of PD.

MATERIALS AND METHODS

Expression plasmids, cell culture and antibodies

Full-length human DJ-1 cDNA was cloned into the mammalian expression plasmid pcDNA3.1-Myc-His (Invitrogen), and point mutations were introduced by PCR-mediated site-directed mutagenesis using the QuickChange kit (Stratagene). Human α-synuclein and ubiquitin cDNAs were cloned into pRK5-HA vector as described previously (20). Expression plasmids for FLAG-tagged human parkin and V5-tagged human Hsp70 were kindly provided by R. Takahashi (RIKEN Brain Science Institute, Japan), HA-tagged mouse CHIP was kindly provided by S. Hatakeyama (Kyushu University, Japan) and FLAG-tagged human DJ-1 was kindly provided by H. Ariga (Hokkaido University,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Japan). A plasmid containing β-galactosidase cDNA was used as a control in all experiments. The integrity of all constructs was confirmed by sequencing.

Human SH-SY5Y neuroblastoma cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were transfected with plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturers recommendations.

Affinity-purified rabbit polyclonal anti-DJ-1 antibodies, DJ-1 (N) and DJ-1 (C), raised to N- and C-terminal epitopes, respectively, have been described previously (33); mouse monoclonal anti-Myc (clone 9E10), anti-Myc-HRP and anti-HA-HRP antibodies were obtained from Roche; mouse monoclonal anti-FLAG (M2), anti-FLAG-HRP, anti-β-tubulin and rabbit polyclonal anti-actin antibodies were obtained from Sigma; mouse monoclonal anti-V5 and anti-V5-HRP antibodies were obtained from Invitrogen; mouse monoclonal anti-parkin (clone PRK8) antibody has been described elsewhere (64); rabbit polyclonal anti-SOD1 antibody was kindly provided by D. Borchelt (Johns Hopkins University); HRP-coupled anti-mouse and anti-rabbit antibodies were obtained from Pierce.

Co-immunoprecipitation and western blotting

For co-immunoprecipitation from cell cultures, SH-SY5Y cells were transfected with 2 μg of each plasmid. After 48 h, cells were washed with cold PBS and harvested in IP buffer [0.5% Triton X-100, 1× Complete mini protease inhibitor cocktail (Roche), in PBS] Lysates were then rotated at 4°C for 1 h followed by centrifugation at 17 500g for 15 min. The supernatant fractions were then combined with 50 μl protein G sepharose 4 fast flow (Amersham), pre-incubated with 5 μg mouse monoclonal anti-myc, anti-FLAG or anti-V5 antibody followed by rotating overnight at 4°C. The protein G sepharose complex was pelleted and washed once with IP buffer supplemented with 500 mM NaCl, twice with IP buffer alone and three times with PBS. Immunoprecipitates or inputs (1% total lysate) were resolved by SDS–PAGE and subjected to western blot analysis. Bands were visualized by enhanced chemiluminescence (Amersham). Quantitation of protein expression was performed using densitometry analysis software (AlphaImager, Alpha Innotech Corp.) and data were analyzed by Student's *t*-test to determine differences between groups.

Where indicated, SH-SY5Y cells were treated for 24 h with MG132 (5 μM; Affiniti Research), hydrogen peroxide (Sigma), MPP^+ (Sigma), SNAP (Calbiochem) or DMSO-PBS as a control where appropriate, prior to harvesting in IP buffer and submitting to IP, as described earlier.

Cross-linking assay

Soluble cell lysates were quantitated using the BCA kit (Pierce) with BSA standards. Equal quantities of lysate were incubated with DSS (Pierce) for 2 h at 4°C. The reaction was quenched by incubation with 50 mM Tris–HCl, pH 7.5 for 15 min at room temperature. Lysates were analyzed by western blotting with anti-myc or anti-DJ-1 (N) antibody. For co-immunoprecipitation experiments, cells were transfected with 2 μg of pcDNA3.1-Myc-DJ-1 and pcDNA3-FLAG-parkin plasmids, and after 48 h, cells were harvested in IP buffer (1% Triton X-100). Equivalent soluble lysates (3 mg total protein) were incubated with 5 mM DSS for 2 h, quenched with 100 mM Tris–HCl, pH 7.5, and IP was performed with an anti-myc antibody. Immunoprecipitates were stringently washed five times with IP buffer supplemented with 500 mM NaCl and once with PBS, and analyzed by western blotting.

Ubiquitination assay

SH-SY5Y cells were transfected with 2 μg of pcDNA3.1-Myc-DJ-1, pRK5-HA-ubiquitin or pcDNA3-FLAG-parkin plasmids. After 48 h, cells were harvested in IP buffer (1% Triton X-100, 1× Complete protease inhibitor cocktail, in PBS) and IP was performed with an anti-myc antibody. Immunoprecipitates were stringently washed five times in IP buffer supplemented with 500 mM NaCl and once with PBS, and submitted to western blotting with anti-HA and anti-myc antibodies. For preparation of detergent-insoluble fractions from cells, the 1% Triton X-100-insoluble pellet fraction was solubilized by sonication and boiling in 100 μl 2× SDS sample buffer containing β-mercaptoethanol. Equivalent detergent-insoluble fractions were analyzed by western blotting.

Human brain tissue

Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions (JHMI) according to HIPAA regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The JHMI Joint Committee on Clinical Investigations decided that the studies in this proposal are exempt from Human Subjects Approval because of Federal Register 46.101 exemption number 4. Tissue from six control brains, two AD brains and five PD/DLB brains were utilized for western blot analysis. Table 1 shows clinical details of subjects used in this study. Frontal cortex tissue from four AR-JP brains was collected at the Department of Neurology and Department of Pathology, Juntendo University School of Medicine, Japan (Table 1). AR-JP cases 1 and 3 each carry a homozygous deletion of parkin exon 4, whereas cases 2 and 4 each carry a homozygous deletion of parkin exon 3, as described previously (7,59,67).

Fractionation experiments

Detergent-soluble and -insoluble fractions were prepared from human brain tissue by homogenization of samples in TNE buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA) containing Complete protease inhibitor cocktail (Roche) and detergent (0.5% NP-40). The homogenate was centrifuged (20 min at 100 000*g*), and the resulting pellet (P1) and supernatant (S1, detergent-soluble) fractions were collected. The P1 fraction was washed once in TNE buffer containing detergent, and the resulting pellet (P2, detergent-insoluble) was homogenized and further solubilized by sonication and boiling in TNE buffer containing 1% SDS

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and 0.5% sodium deoxycholate. Fractions were quantitated using the BCA kit (Pierce) with BSA standards, and analyzed by western blotting.

ACKNOWLEDGEMENTS

The authors would like to thank Drs K. Chung and O. Pletnikova for provision and preparation of human brain fractions. This work was directly supported by grants from the USPHS NS38377 and NS43691, and by grant AG05146 awarded to the Johns Hopkins University (JHU) Alzheimer's Disease Research Center. D.J.M. is supported by the Herbert Freidberg Fellowship. T.M.D. is the Leonard and Madlyn Abramson Professor of Neurodegenerative Diseases at JHU.

REFERENCES

1. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. First of two parts. *N. Engl. J. Med.*, **339**, 1044–1053.
2. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. Second of two parts. *N. Engl. J. Med.*, **339**, 1130–1143.
3. Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M. and Goedert, M. (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl Acad. Sci. USA*, **95**, 6469–6473.
4. Gasser, T. (2003) Overview of the genetics of parkinsonism. *Adv. Neurol.*, **91**, 143–152.
5. Dawson, T.M. and Dawson, V.L. (2003) Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J. Clin. Invest.*, **111**, 145–151.
6. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. *et al.* (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
7. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
8. Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M. *et al.* (2003) Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. *Science*, **299**, 256–259.
9. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G. *et al.* (2004) Hereditary early-onset Parkinson's disease caused by mutations in *PINK1*. *Science*, **304**, 1158–1160.
10. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and Riess, O. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet.*, **18**, 106–108.
11. Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B. *et al.* (2004) The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.*, **55**, 164–173.
12. Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D.S., Hulihan, M., Maraganore, D., Gwinn-Hardy, K., Wszolek, Z., Dickson, D. *et al.* (2004) Comparison of kindreds with parkinsonism and α-synuclein genomic multiplications. *Ann. Neurol.*, **55**, 174–179.
13. Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R. *et al.* (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science*, **302**, 841.
14. Bonifati, V., De Michele, G., Lucking, C.B., Durr, A., Fabrizio, E., Ambrosio, G., Vanacore, N., De Mari, M., Marconi, R., Capus, L. *et al.* (2001) The parkin gene and its phenotype. Italian PD Genetics Study Group, French PD Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Neurol. Sci.*, **22**, 51–52.
15. Mizuno, Y., Hattori, N., Mori, H., Suzuki, T. and Tanaka, K. (2001) Parkin and Parkinson's disease. *Curr. Opin. Neurol.*, **14**, 477–482.
16. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L. and Dawson, T.M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl Acad. Sci. USA*, **97**, 13354–13359.
17. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.*, **25**, 302–305.
18. Imai, Y., Soda, M. and Takahashi, R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.*, **275**, 35661–35664.
19. Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J. (2001) Ubiquitination of a new form of α-synuclein by parkin from human brain: implications for Parkinson's disease. *Science*, **293**, 263–269.
20. Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L. and Dawson, T.M. (2001) Parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat. Med.*, **7**, 1144–1150.
21. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y. and Takahashi, R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of parkin. *Cell*, **105**, 891–902.
22. Staropoli, J.F., McDermott, C., Martinat, C., Schulman, B., Demireva, E. and Abeliovich, A. (2003) Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. *Neuron*, **37**, 735–749.
23. Ren, Y., Zhao, J. and Feng, J. (2003) Parkin binds to α/β tubulin and increases their ubiquitination and degradation. *J. Neurosci.*, **23**, 3316–3324.
24. Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M. *et al.* (2003) The p38 subunit of the aminoacyl-tRNA synthetase complex is a parkin substrate: linking protein biosynthesis and neurodegeneration. *Hum. Mol. Genet.*, **12**, 1427–1437.
25. Tsai, Y.C., Fishman, P.S., Thakor, N.V. and Oyler, G.A. (2003) Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. *J. Biol. Chem.*, **278**, 22044–22055.
26. Huynh, D.P., Scoles, D.R., Nguyen, D. and Pulst, S.M. (2003) The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. *Hum. Mol. Genet.*, **12**, 2587–2597.
27. Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K.I. and Takahashi, R. (2002) CHIP is associated with parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell*, **10**, 55–67.
28. Hague, S., Rogaeva, E., Hernandez, D., Gulick, C., Singleton, A., Hanson, M., Johnson, J., Weiser, R., Gallardo, M., Ravina, B. *et al.* (2003) Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. *Ann. Neurol.*, **54**, 271–274.
29. Abou-Sleiman, P.M., Healy, D.G., Quinn, N., Lees, A.J. and Wood, N.W. (2003) The role of pathogenic DJ-1 mutations in Parkinson's disease. *Ann. Neurol.*, 54, 283–286.
30. Hedrich, K., Djarmati, A., Schafer, N., Hering, R., Wellenbrock, C., Weiss, P.H., Hilker, R., Vieregge, P., Ozelius, L.J., Heutink, P. *et al.* (2004) DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease. *Neurology*, **62**, 389–394.
31. Clark, L.N., Afridi, S., Mejia-Santana, H., Harris, J., Louis, E.D., Cote, L.J., Andrews, H., Singleton, A., Wavrant De-Vrieze, F., Hardy, J. *et al.* (2004) Analysis of an early-onset Parkinson's disease cohort for DJ-1 mutations. *Movement Disord.*, **19**, 796–800.
32. Hedrich, K., Schafer, N., Hering, R., Hagenah, J., Lanthaler, A.J., Schwinger, E., Kramer, P.L., Ozelius, L.J., Bressman, S.B., Abbruzzese, G. *et al.* (2004) The R98Q variation in DJ-1 represents a rare polymorphism. *Ann. Neurol.*, **55**, 145.
33. Moore, D.J., Zhang, L., Dawson, T.M. and Dawson, V.L. (2003) A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. *J. Neurochem.*, **87**, 1558–1567.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

34. Miller, D.W., Ahmad, R., Hague, S., Baptista, M.J., Canet-Aviles, R., McLendon, C., Carter, D.M., Zhu, P.P., Stadler, J., Chandran, J. *et al.* (2003) L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome system. *J. Biol. Chem.*, **278**, 36588–36595.
35. Macedo, M.G., Anar, B., Bronner, I.F., Cannella, M., Squitieri, F., Bonifati, V., Hoogeveen, A., Heutink, P. and Rizzu, P. (2003) The DJ-1^{L166P} mutant protein associated with early onset Parkinson's disease is unstable and forms higher-order protein complexes. *Hum. Mol. Genet.*, **12**, 2807–2816.
36. Gorner, K., Holtorf, E., Odoy, S., Nuscher, B., Yamamoto, A., Regula, J.T., Beyer, K., Haass, C. and Kahle, P.J. (2004) Differential effects of Parkinson's disease-associated mutations on stability and folding of DJ-1. *J. Biol. Chem.*, **279**, 6943–6951.
37. Olzmann, J.A., Brown, K., Wilkinson, K.D., Rees, H.D., Huai, Q., Ke, H., Levey, A.I., Li, L. and Chin, L.S. (2004) Familial Parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function. *J. Biol. Chem.*, **279**, 8506–8515.
38. Tao, X. and Tong, L. (2003) Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. *J. Biol. Chem.*, **278**, 31372–31379.
39. Honbou, K., Suzuki, N.N., Horiuchi, M., Niki, T., Taira, T., Ariga, H. and Inagaki, F. (2003) The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. *J. Biol. Chem.*, **278**, 31380–31384.
40. Wilson, M.A., Collins, J.L., Hod, Y., Ringe, D. and Petsko, G.A. (2003) The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. *Proc. Natl Acad. Sci. USA*, **100**, 9256–9261.
41. Huai, Q., Sun, Y., Wang, H., Chin, L.S., Li, L., Robinson, H. and Ke, H. (2003) Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. *FEBS Lett.*, **549**, 171–175.
42. Lee, S.J., Kim, S.J., Kim, I.K., Ko, J., Jeong, C.S., Kim, G.H., Park, C., Kang, S.O., Suh, P.G., Lee, H.S. *et al.* (2003) Crystal structures of human DJ-1 and *Escherichia coli* Hsp31, which share an evolutionarily conserved domain. *J. Biol. Chem.*, **278**, 44552–44559.
43. Wilson, M.A., St Amour, C.V., Collins, J.L., Ringe, D. and Petsko, G.A. (2004) The 1.8-A resolution crystal structure of YDR533Cp from *Saccharomyces cerevisiae*: a member of the DJ-1/ThiJ/PfpI superfamily. *Proc. Natl Acad. Sci. USA*, **101**, 1531–1536.
44. Kinumi, T., Kimata, J., Taira, T., Ariga, H. and Niki, E. (2004) Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation *in vivo* in human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.*, **317**, 722–728.
45. Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M., Takahashi, K. and Ariga, H. (2004) DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Rep.*, **5**, 213–218.
46. Mitsumoto, A. and Nakagawa, Y. (2001) DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. *Free Radic. Res.*, **35**, 885–893.
47. Mitsumoto, A., Nakagawa, Y., Takeuchi, A., Okawa, K., Iwamatsu, A. and Takanezawa, Y. (2001) Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. *Free Radic. Res.*, **35**, 301–310.
48. Yokota, T., Sugawara, K., Ito, K., Takahashi, R., Ariga, H. and Mizusawa, H. (2003) Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem. Biophys. Res. Commun.*, **312**, 1342–1348.
49. Zhang, Y., Dawson, V.L. and Dawson, T.M. (2000) Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.*, **7**, 240–250.
50. Dawson, T.M. and Dawson, V.L. (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, **302**, 819–822.
51. Sherer, T.B., Kim, J.H., Betarbet, R. and Greenamyre, J.T. (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and α-synuclein aggregation. *Exp. Neurol.*, **179**, 9–16.
52. Sherer, T.B., Betarbet, R., Stout, A.K., Lund, S., Baptista, M., Panov, A.V., Cookson, M.R. and Greenamyre, J.T. (2002) An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered α-synuclein metabolism and oxidative damage. *J. Neurosci.*, **22**, 7006–7015.
53. Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., Fink, A.L. and Di Monte, D.A. (2002) The herbicide paraquat causes up-regulation and aggregation of α-synuclein in mice: paraquat and alpha-synuclein. *J. Biol. Chem.*, **277**, 1641–1644.
54. Lee, S.J. (2003) α-synuclein aggregation: a link between mitochondrial defects and Parkinson's disease? *Antioxid. Redox Signal*, **5**, 337–348.
55. Betarbet, R., Sherer, T.B., Di Monte, D.A. and Greenamyre, J.T. (2002) Mechanistic approaches to Parkinson's disease pathogenesis. *Brain Pathol.*, **12**, 499–510.
56. Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V. and Greenamyre, J.T. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.*, **3**, 1301–1306.
57. Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L. and Dawson, T.M. (2004) S-Nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science*, **304**, 1328–1331.
58. Canet-Aviles, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A. and Cookson, M.R. (2004) The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc. Natl Acad. Sci. USA*, **101**, 9103–9108.
59. Shimura, H., Hattori, N., Kubo, S., Yoshikawa, M., Kitada, T., Matsumine, H., Asakawa, S., Minoshima, S., Yamamura, Y., Shimizu, N. *et al.* (1999) Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. *Ann. Neurol.*, **45**, 668–672.
60. Hering, R., Strauss, K.M., Tao, X., Bauer, A., Woitalla, D., Mietz, E.M., Petrovic, S., Bauer, P., Schaible, W., Muller, T. *et al.* (2004) Novel homozygous p.E64D mutation in DJ1 in early onset Parkinson disease (PARK7). *Hum. Mutat.*, **24**, 321–329.
61. Bonifati, V., Oostra, B.A. and Heutink, P. (2004) Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease. *J. Mol. Med.*, **82**, 163–174.
62. Tofaris, G.K., Layfield, R. and Spillantini, M.G. (2001) α-Synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett.*, **509**, 22–26.
63. David, D.C., Layfield, R., Serpell, L., Narain, Y., Goedert, M. and Spillantini, M.G. (2002) Proteasomal degradation of tau protein. *J. Neurochem.*, **83**, 176–185.
64. Pawlyk, A.C., Giasson, B.I., Sampathu, D.M., Perez, F.A., Lim, K.L., Dawson, V.L., Dawson, T.M., Palmiter, R.D., Trojanowski, J.Q. and Lee, V.M. (2003) Novel monoclonal antibodies demonstrate biochemical variation of brain parkin with age. *J. Biol. Chem.*, **278**, 48120–48128.
65. Rizzu, P., Hinkle, D.A., Zhukareva, V., Bonifati, V., Severijnen, L.A., Martinez, D., Ravid, R., Kamphorst, W., Eberwine, J.H., Lee, V.M. *et al.* (2004) DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia. *Ann. Neurol.*, **55**, 113–118.
66. Neumann, M., Muller, V., Gorner, K., Kretzschmar, H.A., Haass, C. and Kahle, P.J. (2004) Pathological properties of the Parkinson's disease-associated protein DJ-1 in α-synucleinopathies and tauopathies: relevance for multiple system atrophy and Pick's disease. *Acta Neuropathol. (Berl.)*, **107**, 489–496.
67. Hattori, N., Kitada, T., Matsumine, H., Asakawa, S., Yamamura, Y., Yoshino, H., Kobayashi, T., Yokochi, M., Wang, M., Yoritaka, A. *et al.* (1998) Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals. *Ann. Neurol.*, **44**, 935–941.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Movement Disorders
Vol. 20, No. 7, 2005, pp. 887–890
© 2005 Movement Disorder Society

Genetic Brief

Mutation of the Parkin Gene in a Persian Family: Clinical Progression over a 40-Year Period

Jordi Clarimon, PhD,[1]* Janel Johnson, BA,[1] Ruth Djaldetti, MD,[2] Dena Hernandez, MS,[1] Nobutaka Hattori, MD,[3] Hava Sroka, MD,[2] Yael Barhom, MSc,[2] and Andrew Singleton, PhD[1]

[1]*Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA*
[2]*Department of Neurology, Rabin Medical Center, Beilinson Campus, and Felsenstein Research Center, Petah Tiqva, affiliated to the Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel*
[3]*Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan*

Abstract: We report on an Israeli family originating from Iran in which 4 of 7 brothers born from a consanguineous marriage had juvenile Parkinsonism. Linkage analysis of markers covering the autosomal recessive juvenile Parkinsonism (AR-JP, PARK2, *Parkin* gene, OMIM #602544) gene resulted in a maximal logarithm of odds score of 2.18. A homozygous deletion that expanded from exon 4 to exon 6 was identified in all the patients. Significant clinical heterogeneity was present between siblings. © 2005 Movement Disorder Society
Key words: Parkinson's disease; genetics; Parkin; PARK2; clinical heterogeneity

Parkinson's disease, with prevalence close to 2% after age 65, is one of the most frequent neurodegenerative disorders.[1] The classic symptoms include bradykinesia, rigidity, resting tremor, and postural instability. In 1998, Kitada and coworkers[2] identified a novel gene, designated parkin, in which homozygous mutations cause autosomal recessive juvenile Parkinsonism (AR-JP), which typically has an onset before age 40. Since then, many different parkin mutations, ranging from exon rearrangements (deletions and duplications), point mutations, and small deletions have been described in different families from several populations.[3–5] The phenotype of patients with disease caused by parkin mutations is broad but includes dystonia at onset, diurnal fluctuations, slow disease progression, and a good response to levodopa at low doses.[5] We describe an Israeli family with AR-JP due to a parkin mutation.

PATIENTS AND METHODS

Patients

We analyzed a Persian Jewish family who emigrated from Iran to Israel in the early 1950s. There were 4 brothers of 7 siblings born to fist-degree consanguineous parents who had juvenile Parkinsonism (see Fig. 1). Neither parent has Parkinsonism. The affected cases fulfilled the UK Parkinson's Disease Society Brain Bank criteria for disease.[6]

Genetic Analysis

After informed consent was obtained, blood samples were collected from all 4 patients and 3 unaffected brothers, and DNA extraction was performed using standard methods. Genotyping of the microsatellite markers D6S308, D6S441, D6S1581, D6S264, and D6S446 was conducted by the polymerase chain reaction (PCR) using fluorochrome-labeled primers. PCR products were run simultaneously in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

*Correspondence to: Dr. Jordi Clarimon, Molecular Genetics Section, Laboratory of Neurogenetics, National Institutes of Health, Building 35, Room 1A1002, MSC 3707, 35 Lincoln Drive, Bethesda, MD 20892. E-mail: clarimon@mail.nih.gov
Received 2 November 2004; Revised 14 December 2004; Accepted 14 December 2004
Published online 25 April 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.20495

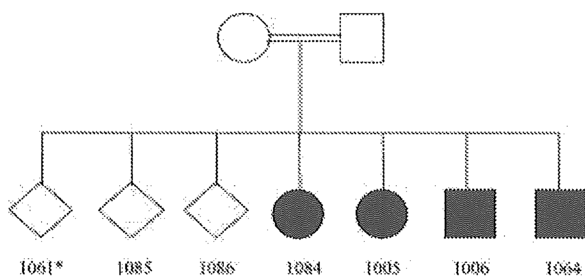

FIG. 1. Pedigree of the Israeli family. Squares represent men, circles represent women. Gender of healthy family members has been masked for confidentiality reasons. Black symbols indicate individuals manifesting autosomal recessive juvenile Parkinsonism. The healthy sibling carrying a heterozygous deletion within the *Parkin* gene is depicted by an asterisk. All the patients carried a homozygous deletion of the exons 4 through 6.

We performed two-point linkage analysis using a parametric model with a recessive mode of inheritance, a frequency of the susceptibility allele of 0.01%, and a homozygous penetrance of 100%. Pair-wise linkage calculations were performed using MLINK.

Testing for gene dosage alterations in *Parkin* exons was carried out by quantitative duplex PCR using the ABI PRISM 7900 sequence detection system (Applied Biosystems). The β-globin gene was coamplified with *Parkin* exons 1 through 12 and served as endogenous reference as previously described.[7] The dosage of *Parkin* exons relative to β-globin and normalized to control DNA was determined using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Molecular Findings

The two-point maximum logarithm of odds score was 2.18 for markers D6S264 and D6S446 at a theta = 0. These markers are located 3 megabases (MB) and 7 MB, respectively, from the *Parkin* gene, thus indicating a PARK2 mutation segregating with the disease.

Real-time quantitative PCR-based gene dosage assays evidenced homozygous deletion of exons 4 to 6 in all the patients. Only 1 of the healthy siblings (individual 1061) carried a heterozygous deletion. The other 2 siblings did not harbor a deletion.

Clinical Description

Clinical characteristics are shown in Table 1.

Patient 1084.

On her first examination (at which time she was 28 years old), the patient had hypomimia, rest tremor in all extremities and in the tongue, mild rigidity, and bradykinesia in all limbs. Treatment with anticholinergic agents was initiated with beneficial response. Nine years later, she started treatment with L-dopa/carbidopa (125 mg/day) with marked response. At the age of 38, she developed oral and limb dyskinesias. She could not tolerate either the combination of L-dopa/carbidopa (125 mg t.i.d.) or a high dose of L-dopa and, until present, is treated with pure L-dopa (100 mg/day). After a disease duration of 35 years, severe freezing is reported with recurrent falls, yet without postural instability. At that time, she was still L-dopa–responsive with wearing off fluctuations. Other medications are selegiline 5mg/day, and trihexyphenidyl 5 mg/day. She could not tolerate dopamine agonists (pergolide and ropinirole). Over the next years, her symptoms slowly progressed. At the time of her most recent examination, she had marked difficulties with walking, severe freezing gait, marked rigidity, and dyskinesias of the left foot.

Patient 1064.

The patient started treatment with trihexyphenidyl with good response. During the first years of the disease, there were no other major complaints besides depression and anxiety. Twelve years after disease onset, he complained of migrating pain in different parts of the body, constipation, depression, and insomnia. He also could not tolerate carbidopa and was treated with low doses of L-dopa (100 mg/day) with moderate response. He remained stable over the course of 10 years and even stopped taking L-dopa, as he felt worsening of his symptoms (pain, tremor, general weakness) under all the

TABLE 1. *Patient clinical characteristics*

	Patient 1084	Patient 1005	Patient 1006	Patient 1064
Age at onset (yr)	18	21	19	39
Age at last examination (yr)	67	62	65	67
Initial symptom	Legs rest tremor	Legs rest tremor	Postural instability	Right extremities rest tremor
Dystonia	+	–	+	+
Psychiatric symptoms	–	–	–	+
Diurnal fluctuations	+	–	+	–
Slow progression	+	+	+	+

L-dopa preparations. He was treated only with trihexyphenidyl and selegiline 5 mg/day. The most prominent symptoms were psychiatric, with psychomotor restlessness, marked anxiety, akathisia, and unexplained fears.

Patient 1006.

At age 32, he was first examined at our clinic. His main complaints were fatigue and excessive sweating. On examination, head and limb tremor was noticed, more on the left side of the body, mild rigidity, and marked retropulsion. At that time, L-dopa treatment was initiated with amelioration of the tremor and improvement of postural instability. Three years later motor fluctuations, dyskinesias, and dystonia of the right foot emerged. The patient presented intolerance to carbidopa and responded to very low doses of L-dopa (200 mg/day). The parkinsonian symptoms progressed slowly, but 31 years after disease onset, he became very disabled due to freezing gait with recurrent falls. On his recent examination, he was still taking very low doses of pure L-dopa (50 mg q.i.d), selegiline 5 mg/day, trihexyphenidyl 5 mg/day, and amantadine 200 mg/day. On examination while *on*, the patient had hypomimia, dysarthria, no tremor, mild rigidity in all extremities, was able to arise from chair with assistance, and gait was impaired with freezing episodes and postural instability. While *off*, he had marked hypomimia, rigidity, and bradykinesia of all four limbs, and he was almost akinetic, with severe and frequent freezing episodes.

Patient 1005.

Eleven years from disease onset, low doses of L-dopa were initiated with very good response. During the course of the disease there were diurnal variations without sleep benefit, as she felt worse in the morning hours. Her symptoms included fatigue, weight loss, excessive sweating, and pain on the right hand. On her last examination, the most prominent symptom was rest and postural tremor of all extremities and increased tonus more on the right side of the body. Hand and leg movements were normal. She had no difficulties rising from a chair, and there was no gait abnormality.

DISCUSSION

We report on an AR-JP family with disease duration of more that 40 years. Gene dosage analysis revealed an exon 4 to 6 homozygous deletion in *Parkin*. We did not sequence the entire gene and, therefore, cannot rule out any further point mutations in this family.

This mutation has been described also by other authors[8–10]and, as far as we know, this is the third deletion in the *Parkin* gene described in an Israeli family suffering from AR-JP. The same deletion extent was described in two Israeli brothers with first-degree cousin parents.[10] Although both siblings had good response to L-dopa and presented dystonia during the course of the disease, it is noteworthy that none of them exhibited resting tremor. Another Jewish Yemenite kindred also has been described to harbor an exon 3 homozygous deletion.[11] Finally, a single base pair deletion at nucleotide 202 in exon 2 of the *Parkin* gene also has been identified in another Arabic Muslim Israeli family.[12] These studies demonstrate the existence of allelic and phenotypic heterogeneity of *Parkin* mutations in the Israeli population.

The patients described in the present study had beneficial response to L-dopa, slow progression of the disease, and diurnal fluctuations, common among patients with *Parkin* mutations. Our cases share similar clinical features to other parkin cases, including age at onset, symptoms at disease onset, beneficial response to L-dopa, and the occurrence of nonmotor symptoms. However, some features are unique to this kindred: all members have hypersensitivity to L-dopa, responding dramatically to very low doses of L-dopa (at this time, they are treated with strikingly low doses of L-dopa, between 50–100 mg b.i.d. or q.i.d.), and attempts to increase the dose resulted in general complaints; a wide interval of age at onset, ranging from 18 to 39 years, is also presented; psychiatric symptoms seem to be present in only 1 of the 4 patients; and an asymmetrical motor symptom at onset is also reported only by Patient 1064. Dystonia, a common sign in AR-PD patients, was not present in Patient 1005, whereas the other siblings presented different dystonic signs. This phenotypic heterogeneity could be due to the interaction of *Parkin* with other genes (epistasis) and/or other environmental effects.

Acknowledgment: We thank the participating patients for their cooperation.

REFERENCES

1. de Rijk MC, Tzourio C, Breteler MM, et al. Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. J Neurol Neurosurg Psychiatry 1997;62:10–15.
2. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605–608.
3. West AB, Maidment NT. Genetics of parkin-linked disease. Hum Genet 2004;114:327–336.
4. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999;8:567–574.
5. Ujike H, Yamamoto M, Kanzaki A, Okumura K, Takaki M, Kuroda S. Prevalence of homozygous deletions of the parkin gene

in a cohort of patients with sporadic and familial Parkinson's disease. Mov Disord 2001;16:111–113.
6. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181–184.
7. Dogu O, Johnson J, Hernandez D, et al. A consanguineous Turkish family with early-onset Parkinson's disease and an exon 4 parkin deletion. Mov Disord 2004;19:812–816.
8. Nichols WC, Pankratz N, Uniacke SK, et al. Linkage stratification and mutation analysis at the Parkin locus identifies mutation positive Parkinson's disease families. J Med Genet 2002;39:489–492.
9. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003;60:796–801.
10. Portman AT, Giladi N, Leenders KL, et al. The nigrostriatal dopaminergic system in familial early onset parkinsonism with parkin mutations. Neurology 2001;56:1759–1762.
11. Nisipeanu P, Inzelberg R, Blumen SC, et al. Autosomal-recessive juvenile parkinsonism in a Jewish Yemenite kindred: mutation of Parkin gene. Neurology 1999;53:1602–1604.
12. Nisipeanu P, Inzelberg R, Abo Mouch S, et al. Parkin gene causing benign autosomal recessive juvenile parkinsonism. Neurology 2001;56:1573–1575.

Preserved Myocardial [^{123}I]Metaiodobenzylguanidine Uptake in Autosomal Recessive Juvenile Parkinsonism: First Case Report

Masahiko Suzuki, MD, PhD,[1*] Nobutaka Hattori, MD, PhD,[2] Satoshi Orimo, MD,[3] Nobuyoshi Fukumitsu, MD,[4] Masahiro Abo, MD, PhD,[5] Yu Kono, MD,[1] Renpei Sengoku, MD,[1] Akira Kurita, MD, PhD,[1] Hidehiko Honda, MD, PhD,[1] and Kiyoharu Inoue, MD, PhD[1]

[1]*Department of Neurology, The Jikei University School of Medicine, Tokyo, Japan*
[2]*Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan*
[3]*Department of Neurology, Kanto Central Hospital, Tokyo, Japan*
[4]*Department of Radiology, The Jikei University School of Medicine, Tokyo, Japan*
[5]*Department of Rehabilitation Medicine, The Jikei University School of Medicine, Tokyo, Japan*

Abstract: A decrease in myocardial uptake of iodine-123–labeled metaiodobenzylguanidine (^{123}I-MIBG) has been reported in idiopathic Parkinson's disease (PD) using ^{123}I-MIBG myocardial scintigraphy. However, the patient with autosomal recessive juvenile parkinsonism (AR-JP), caused by the *parkin* gene, presented here showed normal ^{123}I-MIBG myocardial uptake, suggesting that AR-JP is a distinct disease entity from PD. Although the clinical features of AR-JP are sometimes quite similar to those of late-onset idiopathic PD, ^{123}I-MIBG myocardial scintigraphy may be a powerful tool to differentiate PD from other parkinsonian syndromes, including AR-JP. © 2005 Movement Disorder Society

Key words: autosomal recessive juvenile parkinsonism; Parkinson's disease; metaiodobenzylguanidine

Myocardial scintigraphy using iodine-123–labeled meta-iodobenzylguanidine (^{123}I-MIBG) is used to differentiate idiopathic Parkinson's disease (PD) from other neurological diseases.[1–4] Low myocardial ^{123}I-MIBG uptake is a consistent finding in PD.[1–3] A recent neuropathological study suggests that low ^{123}I-MIBG uptake indicates the involvement of postganglionic sympathetic function in PD.[5]

Recently, autosomal recessive juvenile parkinsonism (AR-JP) has been found to be caused by the *parkin* gene.[6] Although the clinical features of AR-JP are sometimes quite similar to those of late-onset idiopathic PD, neuropathological studies have revealed no Lewy bodies in the substantia nigra and locus ceruleus of most cases of AR-JP, suggesting that the pathological findings and disease process of AR-JP are different from those of PD.[7,8] In the present study, we investigated cardiac sympathetic function in a patient with AR-JP using ^{123}I-MIBG scintigraphy and evaluated the clinical applicability of this method.

Case Report

The patient was a 62-year-old, Japanese man with a 32-year history of parkinsonism. He was the only son of consanguineous parents from Niigata Prefecture, Japan. At 30 years of age, dystonic posture of the legs and gait disturbance developed. At presentation, the clinical features included diurnal fluctuation and slow progression of the disease. The parkinsonian triad was mild, and the tremor was usually of the fine type. These clinical features markedly improved during sleep. Treatment with levodopa effectively improved the condition, which exhibited a benign clinical course. He was treated with 500 mg of L-dopa/carbidopa and 22.5 mg of bromocriptine per day, which resulted in marked improvement in bradykinesia and rigidity. However, the wearing-off phenomenon was marked, and the response to L-dopa gradually decreased. AR-JP was diagnosed on the basis of the medical history, physiological and neurological examinations, routine laboratory tests, magnetic resonance imaging studies of the brain, and *parkin* gene mutation analysis, including quantitative analysis using real-time polymerase chain reaction (PCR) to detect a compound heterozygote (Fig. 1).[9] Results of quantitative PCR amplification analysis showed that the relative dose value for exons 2 to 4 was approximately 0.5, indicating that this patient was a compound heterozygote for exons 2 and 3 to 4 deletions or exons 2 to 3 and 4 deletions. Alternatively, it is possible that this patient was a heterozygote for deletions of exons 2 to 4. Considering the clinical information, including the age of onset and the mode of

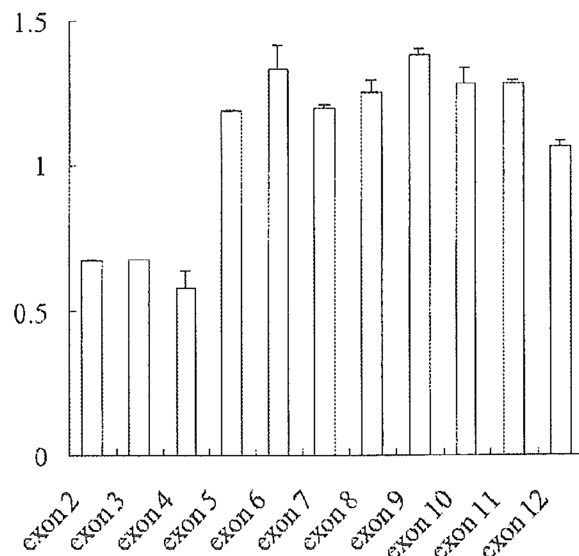

FIG. 1. Real-time polymerase chain reaction shows heterodeletion in exons 2, 3, and 4, indicating compound heterozygosity. The vertical axis represents the ratio of each exonic dose of the *parkin* gene to the dose of the β-actin gene.

*Correspondence to: Dr. Masahiko Suzuki, Department of Neurology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan. E-mail: suzukimd@jikei.ac.jp

Received 8 March 2004; Revised 19 August 2004; Accepted 21 September 2004

Published online 8 February 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.20384

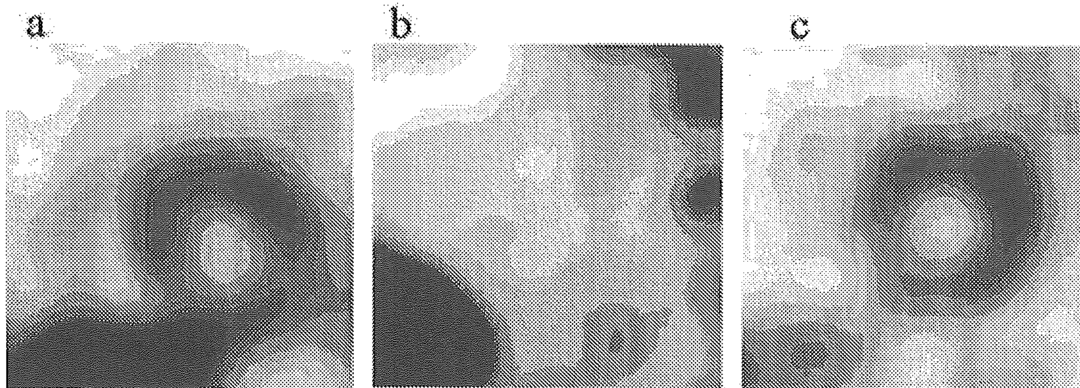

FIG. 2. Short-axis views in the early phase of single-photon emission computed tomography of iodine-123–labeled metaiodobenzylguanidine (^{123}I-MIBG) myocardial scintigraphy in a 62-year-old man with autosomal recessive juvenile parkinsonism (**a**), a 58-year-old man with idiopathic Parkinson's disease (Hoehn and Yahr Stage II, **b**), and a healthy 62-year-old man (**c**).

inheritance, the patient may be a compound heterozygote rather than heterozygote for a deletion of exons 2 to 4.

Single-photon emission computed tomography (SPECT) ^{123}I-MIBG scintigraphy of the heart was performed with an intravenous injection of 111 MBq of ^{123}I-MIBG (Daiichi Radioisotope Laboratories Co., Tokyo, Japan). Early and delayed SPECT was obtained 30 minutes and 4 hours after injection, respectively. Short-axis reconstruction of ^{123}I-MIBG early SPECT acquisitions showed normal ^{123}I-MIBG uptake (Fig. 2). The mean values of the heart to mediastinum (H/M) ratio in the early and delayed phases were 1.99 and 2.13, respectively. In our hospital, the same ratios in age-matched healthy control subjects ($n = 19$) were early phase, 2.02 ± 0.12 (range, 1.86–2.25), delayed phase, 2.05 ± 0.15 (range, 1.85–2.38).

Discussion

To our knowledge, this is the first report of ^{123}I-MIBG myocardial scintigraphy in a patient with AR-JP. Myocardial uptake of ^{123}I-MIBG was normal in this patient (Fig. 2). The H/M ratios in the early and delayed phases in this patient were well within the range of healthy control subjects, although the disease duration was more than 30 years. These findings might explain the tendency for cardiac sympathetic function to be normal in patients with AR-JP.

In PD, ^{123}I-MIBG uptake of postganglionic cardiac sympathetic neurons is reduced at an early stage of the disease in almost all patients with a clinical severity score of Hoehn and Yahr stage II or higher.[1,3] In addition, ^{123}I-MIBG uptake was reduced even in patients whose symptoms had been present less than 5 years, although the reduction was significant in patients with advanced disease.[1] The pathological background of PD is a systemic distribution of Lewy bodies and Lewy neuritis, spreading to the peripheral autonomic nervous system, including the cardiac plexus.[10,11] However, neuropathological studies in AR-JP have revealed selective degeneration with gliosis of the pigmented neurons of the substantia nigra and locus ceruleus, but generally no Lewy bodies,[7,8,12] suggesting that the pathological findings and disease process of AR-JP differ from those of PD. Considered together, our findings in AR-JP and those of previous studies in PD allow us to speculate that AR-JP might be a disease entity distinct from PD, although the clinical features of both diseases are quite similar.

Although this study consisted of only 1 patient with AR-JP, our findings indicate that cardiac sympathetic nerve denervation occurs in PD, which accounts for the decrease in cardiac uptake of ^{123}I-MIBG, but not in AR-JP. In this regard, quantification of cardiac ^{123}I-MIBG uptake has been considered a valuable tool to identify patients with PD and to distinguish them from patients with other parkinsonian syndromes, including AR-JP.[1–4] Further studies involving ^{123}I-MIBG uptake and neuropathological examination of the sympathetic nervous system should be performed in a larger population of patients with *parkin* mutations to confirm our conclusion.

References

1. Orimo S, Ozawa E, Nakade S, Sugimoto T, Mizusawa H. ^{123}I-metaiodobenzylguanidine myocardial scintigraphy in Parkinson's disease. J Neurol Neurosurg Psychiatry 1999;67:189–194.
2. Braune S, Reinhardt M, Schnitzer R, Riedel A, Lucking CH. Cardiac uptake of [^{123}I]MIBG separates Parkinson's disease from multiple system atrophy. Neurology 1999;53:1020–1025.
3. Druschky A, Hilz MJ, Platsch G, et al. Differentiation of Parkinson's disease and multiple system atrophy in early disease stages by means of I-123-MIBG-SPECT. J Neurol Sci 2000;175:3–12.
4. Taki J, Nakajima K, Hwang EH, et al. Peripheral sympathetic dysfunction in patients with Parkinson's disease without autonomic failure is heart selective and disease specific. Eur J Nucl Med 2000;27:566–573.
5. Orimo S, Oka T, Miura H, et al. Sympathetic cardiac denervation in Parkinson's disease and pure autonomic failure but not in multiple system atrophy. J Neurol Neurosurg Psychiatry 2002;73:776–777.
6. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605–608.
7. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51:890–892.
8. Paviour DC, Surtees RA, Lees AJ. Diagnostic considerations in juvenile parkinsonism. Mov Disord 2004;19:123–135.
9. Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. Pseudo-autosomal dominant inheritance of PARK2: two families with parkin gene mutations. J Neurol Sci 2003;207:11–17.

10. Wakabayashi K, Takahashi H. Neuropathology of autonomic nervous system in Parkinson's disease. Eur Neurol 1997;38(Suppl. 2):2–7.
11. Iwanaga K, Wakabayashi K, Yoshimoto M, et al. Lewy body-type degeneration in cardiac plexus in Parkinson's and incidental Lewy body diseases. Neurology 1999;52:1269–1271.
12. Takahashi H, Ohama E, Suzuki S, et al. Familial juvenile parkinsonism: clinical and pathologic study in a family. Neurology 1994;44:437–441.

Feasibility and Validity of a Modified Finger-Nose-Finger Test

Elan D. Louis, MD, MS,[1–3*] LaKeisha M. Applegate, BA,[1] Sarah Borden, BA,[1] Carol Moskowitz, MS,[2] and Zhezhen Jin, PhD[4]

[1]*G.H. Sergievsky Center, College of Physicians and Surgeons, Columbia University, New York, New York, USA*
[2]*Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, New York, USA*
[3]*Taub Institute for Research on Alzheimer's Disease and the Aging Brain, College of Physicians and Surgeons, Columbia University, New York, New York, USA*
[4]*Department of Biostatistics, Mailman School of Public Health, Columbia University, New York, New York, USA*

Abstract: In essential tremor (ET) research, it is important to obtain standardized, objective data on tremor severity. Often, it is not possible to carry out in-person or videotaped neurological examinations. In place of these, handwriting samples can be collected, but they do not capture all of the variance in tremor severity. Although additional tests of tremor severity (finger-nose-finger [FNF] test) might be of use, these would need to be modified to allow ET patients to mail their results to the study investigator for rating. We modified the standard FNF test (sFNF) by asking subjects to hold a pen during this activity and mark a paper target. The purpose of this report was to determine whether the modified FNF (mFNF) test was feasible and valid. Of 70 subjects, 65 (92.9%) were able to complete the mFNF, demonstrating that it was feasible. The scores of the mFNF correlated highly with those of the sFNF ($r = 0.56$–0.85; all $P < 0.001$), indicating the mFNF is a valid measure of tremor severity. In addition, using the regression equation, sFNF = 0.174(mFNF) + 0.743, a sFNF score can be derived easily from the mFNF score. The mFNF may be used to collect valuable data on tremor severity in pathological, genetic, and epidemiological field studies of ET, in which in-person or videotaped neurological examinations are not possible. © 2005 Movement Disorder Society

Key words: essential tremor; epidemiology; validity; brain bank; screen

Essential tremor (ET) patients from throughout the United States currently are being recruited prospectively as potential brain donors to the Essential Tremor Centralized Brain Repository at Columbia University.[1] Over-arching goals of the repository are to identify the pathology of ET and to study the clinical correlates of that pathology (i.e., comparing the severity and laterality of clinical with pathological findings). It is imperative, therefore, to obtain standardized, objective data on tremor severity. Although an in-person examination or a visual record (videotaped examination) is ideal for this purpose, ET patients are scattered geographically throughout the United States and most do not have access to a videocamera. This problem is also encountered commonly in epidemiological and family studies of ET, and this provides the impetus to explore alternative objective methods. One approach is for the ET patients to mail handwriting samples to the investigators at the Brain Bank. Although these would be of some use, they would not capture all of the variance in tremor severity; tremor while writing can be minimal when compared with tremor during other actions (e.g., finger-nose-finger [FNF] test)[2]; alternatively, in cases of primary writing tremor, the converse is true.[3] In an attempt to devise additional methods for assessing tremor severity, we modified the FNF test by asking the subject to hold a pen during this activity and to mark a paper target. The purpose of this report was to determine the feasibility and validity of this method. If the method were valid, it could be used in this and other brain bank studies as well as in epidemiological and genetic studies to collect objective data on tremor severity.

Subjects and Methods

Subjects

We assessed the modified FNF (mFNF) test in individuals with a broad representation of tremor severities, ranging from severe action tremor to minimal or no action tremor. We enrolled individuals in three diagnostic groups, including individuals who were expected to have: (1) moderate to severe action tremor (patients with ET); (2) minimal or no action tremor (normal controls); and (3) intermediate amounts of action tremor, i.e., patients with Parkinson's disease (PD).[4] Our goal was to enroll 20 to 30 subjects per group (total sample size = 75), which would provide adequate power to address our aims.

As described previously,[5,6] ET patients and controls were participants in an epidemiological study. These ET patients were referred to the study by neurologists at the Neurological Institute of New York, Columbia University Medical Center (CUMC) and all controls were identified from the New York Metropolitan area using random digit telephone dialing as described previously.[5,6] Each subject had an in-person assessment, which was carried out by a trained research assistant. The assessment included a videotaped neurological examination with the standard FNF (sFNF) test. The data used in the current report were derived from ET patients and controls who were

This article includes Supplementary Video, available online at http://www.interscience.wiley.com/jpages/0885-3185/suppmat.

*Correspondence to: Dr. Elan Louis, Unit 198, Neurological Institute, 710 West 168th Street, New York, NY 10032.
E-mail: EDL2@columbia.edu

Received 13 July 2004; Revised 1 September 2004; Accepted 22 September 2004

Published online 16 February 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.20409

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 331 (2005) 309–317

BBRC

www.elsevier.com/locate/ybbrc

Phosphorylated IκBα is a component of Lewy body of Parkinson's disease

Kazuyuki Noda [a], Toshiaki Kitami [a], Wei Ping Gai [c], Fariba Chegini [c], Poul Henning Jensen [d], Tsutomu Fujimura [b], Kimie Murayama [b], Keiji Tanaka [e], Yoshikuni Mizuno [a], Nobutaka Hattori [a,*]

[a] *Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Division of Biochemical Analysis, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[c] *Department of Human Physiology and Center for Neuroscience, Flinders University, Bedford Park, SA5042, Australia*
[d] *Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*
[e] *Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan*

Received 20 March 2005
Available online 2 April 2005

Abstract

Ubiquitin is one of the major components of Lewy bodies (LB), the pathological hallmark of Parkinson's disease (PD). Here, we identified that a phosphorylated form of IκBα (pIκBα), an inhibitor of NF-κB, and $SCF^{\beta\text{-TrCP}}$, the ubiquitin ligase of pIκBα, are components of LB in brains of PD patients. In vitro studies identified those proteins in the ubiquitin- and α-synuclein (known as the major component of LB)-positive LB-like inclusions generated in dopaminergic SH-SY5Y cells treated with MG132, a proteasome inhibitor. Intriguingly, IκBα migration into such ubiquitinated inclusions in cells treated with MG132 was inhibited by a cell-permeable peptide known to block phosphorylation of IκBα, although this peptide did not influence cell viability under proteasomal inhibition. Our results indicate that phosphorylation of IκBα plays a role in the formation of IκBα-containing inclusions caused by proteasomal dysfunction, and that the generation of such inclusion is independent of cell death caused by impairment of proteasome.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Parkinson's disease; Lewy bodies; Ubiquitin; Proteasome; IκBα; IκB-kinase

Parkinson's disease (PD) is one of the most common neurodegenerative disorders among the aged and its pathological hallmark is the preferential degeneration of dopaminergic neurons in the substantia nigra (SN) and the appearance of intracytoplasmic inclusions known as Lewy bodies (LB). The process of LB formation could provide important clues regarding the pathogenesis of PD because important proteins, such as α-synuclein (another familial PD gene product) and ubiquitin (Ub), are components of these inclusions [1,2].

The ubiquitin–proteasome pathway (UPP) is the major non-lysosomal degradation system for various proteins, such as short-lived, misfolded, and damaged polypeptides [3]. In this system, ubiquitin is conjugated to lysine residue of the target protein by a cascade of enzymatic reactions catalyzed by the E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes in an ATP-dependent manner, and polyubiquitination marks the proteins for degradation by the proteasome. Several lines of evidence suggest that derangements in the UPP play an important role in the pathogenesis of PD and describe inhibited hydrolytic activities of the proteasome in PD [4,5].

* Corresponding author. Fax: +81 3 5800 0547.
E-mail address: nhattori@med.juntendo.ac.jp (N. Hattori).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.167

On the other hand, the concentration of tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is increased in PD and this cytokine plays a role in the pathogenesis of PD [6,7]. TNF-α stimulates the multisubunit IκB-kinase (IKK), which is composed of IKKα, IKKβ and a non-catalytic regulatory component named NF-κB essential modifier (NEMO), which allows IκBα to be subsequently phosphorylated. The phosphorylated form, pIκBα, is polyubiquitinated by the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase [8–10]. Subsequently, they are rapidly degraded by 26S proteasomes, and NF-κB enters the nucleus, binds to DNA, and activates transcription of target genes [11]. In this scenario, the nuclear translocation of NF-κB is masked by physical association with IκBα, resulting in retention of NF-κB in the cytoplasm.

Intriguingly, a previous report implicated the involvement of NF-κB in the LB of PD [12], but the role of IκBα in the pathogenesis of PD remains unclear. The aims of the present study were the following: (1) to determine the presence or absence of pIκBα and components of the $SCF^{\beta\text{-TrCP}}$ complex in LB of PD, (2) whether pIκBα is colocalized in the cytoplasmic inclusions formed in MG132-treated human dopaminergic neuroblastoma cells (SH-SY5Y), and (3) to determine the effect of inhibition of IκBα phosphorylation on the formation of cytoplasmic inclusions and viability of cells treated with a proteasome inhibitor.

Materials and methods

Human neuroblastoma cell lines. Human dopaminergic neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. To induce differentiation, the cells were treated with 10 μM retinoic acid (Sigma Chemical, St. Louis, MO) in the dark for 4–6 days as described previously [13–15].

Application of agents. TNF-α (R&D Systems, Minneapolis, MN) and MG132 (Peptide Institute, Osaka, Japan) were prepared at 10 μg/ml (in H_2O) and 10 mM (in dimethyl sulfoxide, DMSO) stock solutions, respectively. The specific antibodies used were rabbit anti-ubiquitin (Dako, Carpinteria, CA), mouse anti-ubiquitin 1510 (Chemicon International, Temecula, CA), anti-synuclein-1 (Transduction Laboratories, Lexington, KY), sheep anti-α-synuclein [16], anti-pIκBα (Ser32), and anti-IκBα antibodies (from Cell Signaling Technology, Beverly, MA, and Calbiochem, La Jolla, CA). Anti-NF-κB p65 (sc-372) and anti-β-TrCP (N-15) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Note that anti-IκBα antibody reacts with both phosphorylated and unphosphorylated forms of IκBα. Anti-ROC1 and Cul-1 antibodies were prepared as described previously [17]. The secondary antibodies used were goat anti-mouse IgG coupled with Alexa Fluor 488, goat anti-mouse IgG coupled with Alexa Fluor 594, anti-rabbit IgG coupled with Alexa Fluor 594 (Molecular Probes, Eugene, OR), and fluorescein goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

Immunohistochemistry. For the LB staining experiments using paraffin embedded samples, autopsied brains from seven patients with PD (age, 51–78 years), one patient with dementia with Lewy bodies (DLB, age, 64 years), and five control subjects (age, 20–65 years) were examined. The five control samples were obtained from patients free of neurological diseases and confirmed to have no neuropathological changes in sections of the substantia nigra pars compacta. Immunohistochemistry was performed as described previously [18].

Immunolabeling of isolated Lewy bodies. Immunomagnetic isolation and immunostaining of LB and Lewy neuritis from fresh frozen brains of patients with DLB were performed as described previously [19]. Smears were prepared on gelatin-coated glass slides, from homogenates, factions of each washing step, and LB-enriched Percoll fractions. The smears were air-dried overnight, fixed for 10 min in 4% formaldehyde–2% picric acid–0.1 M phosphate-buffered saline (pH 7.4), and then incubated for 10 min in 3% H_2O_2 in 50% methanol in Tris-buffered saline (TBS, pH 7.4). Following three rinses in 0.1 M TBS containing 0.05% sodium azide and 1 mM phenylmethylsulfonyl fluoride (PMSF) (TBS), the smears were incubated with 20% normal horse serum in TBS for 30 min to block non-specific antibody-binding sites, and incubated overnight with sheep antibody against α-synuclein and pIκBα, ROC1, Cul-1, and β-TrCP in TBS containing 0.5% bovine serum albumin. Control sections were stained by incubating the smears with TBS containing either or neither antibodies. Following three rinses in TBS, the smears were incubated for 1 h with donkey anti-sheep IgG or donkey anti-rabbit IgG conjugated with Cy2, Cy3 or Cy5 in TBS (all from Jackson Immunoresearch Laboratories, West Grove, PA), with the fluorochromes either singly or in combinations of Cy2/Cy3 or Cy2/Cy5.

Immunocytochemistry. For double-labeling immunofluorescence staining, fixed cells were permeabilized with 0.25% Triton X-100 for 20 min. The cells were blocked with 5% normal goat serum for 1 h, followed by incubation of antibodies to ubiquitin (Chemicon; diluted 1:100), pIκBα Ser32 (Cell Signaling; diluted 1:100), ROC1 (diluted 1:100), or Cullin-1 (diluted 1:100) for 1 h at room temperature. After washing in TBS, the cells were incubated in anti-mouse, anti-rabbit fluorochrome-linked secondary antibodies. After washing in TBS, the cells were covered with glass slides using mounting medium with 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei (Vector Laboratories). To assess the colocalization of ubiquitin and α-synuclein, we used anti-ubiquitin (Dako; diluted 1:100) and anti-synuclein-1 (diluted 1:100) antibodies. Signal was observed under a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, NY).

Western blotting. Neuronally differentiated SH-SY5Y cells were treated for 24 h with 10 μM MG132 or 20 ng/ml TNF-α. The cells were simultaneously preincubated for 2 h with 10 μM MG132, followed by treatment for 22 h with 10 μM MG132 in the presence of 20 ng/ml TNF-α. The cells were collected and washed in ice-cold phosphate-buffered saline (PBS) and lysis buffer as described previously [20]. Detergent-insoluble material was pelleted by centrifugation at 100,000*g* for 20 min and resuspended in 50 mM Tris–HCl (pH 7.4) and 6 M urea. Equal amounts of protein from both fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% or 10–20%) and transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in 5% non-fat milk for 1 h at room temperature, and incubated at 4 °C with specific antibodies.

Treatment of IKK inhibiting peptide. Recent studies have reported that an NH_2-terminal α-helical region of NEMO associates with a hexapeptide sequence within the extreme carboxyl terminus of IKKβ and IKKα, termed NEMO-binding domain (NBD). Importantly, a short cell-permeable peptide spanning the IKKβ NBD was found to disrupt the association of NEMO with IKKβ and blocked the activity of IKK [21]. We synthesized two peptides at Juntendo University School of Medicine, a functional wild-type NBD with a sequence derived from the Antennapedia homeodomain that mediates membrane translocation without loss of cell viability, and a negative control mutant NBD, as described previously [21–24]. To determine how inhibition of phosphorylation of IκBα affects the formation of such inclusions, the differentiated SH-SY5Y cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 10 μM MG132 in the presence of 40 μM of each NBD peptide. The cells were simultaneously preincubated for 3 h

with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. These cells with ubiquitin-positive inclusions were co-stained with antibodies against pIκBα or IκBα.

Cell viability assay. Cell viability assay was performed using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt assay, as described previously [25]. The differentiated SH-SY5Y cells were plated on a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2. The cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 40 μM of each NBD peptide in the presence or absence of 10 μM MG132. The cells were simultaneously preincubated for 3 h with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. The live cell count was assayed using Cell Counting Kit-8 according to the instructions provided by Doujin (Cell Counting Kit-8; Kumamoto, Japan).

Statistical analysis. All data are expressed as means ± SEM. Comparisons between groups were performed using analysis of variance (Tukey's multiple *t* test).

A p value <0.05 indicated statistically significant differences.

Results

Phosphorylated IκBα and $SCF^{\beta\text{-}TrCP}$ complex are novel components of Lewy bodies

We first examined whether LB contain pIκBα and the components of $SCF^{\beta\text{-}TrCP}$ complex, which are major downstream components of the TNF-α signaling pathway. Immunohistochemical analysis revealed that anti-pIκBα and ROC1 antibodies predominantly recognized the LB in PD cases (Figs. 1A and B). Immunostaining with anti-NF-κB p65 antibody also showed the staining of LB (Fig. 1C). Anti-pIκBα and anti-ROC1 signals were strongly present in the halo region of LB, and the anti-NF-κB-p65 signal was present in the core region. In contrast, such immunoreactivities for pIκBα ROC1 and NF-κB were not observed in the control brains and when the primary antibody was omitted in PD and DLB brains (data not shown).

In the next step, the isolated LB were used to investigate whether these proteins are associated with LB. Confocal laser-scanning microscopic examination of sections prepared from freshly isolated LB from postmortem brains of DLB showed immunoreactivities for pIκBα, ROC1, Cul-1, and β-TrCP (Figs. 1D, G, J, and M). LB were identifiable by their strong α-synuclein staining in smears of isolated LB from DLB cortex (Figs. 1E, H, K, and N), but not in sections from a normal control cortex (data not shown). These immunoreactivities for the indicated antibodies in LB were distributed across or sometimes more concentrated in the central region of LB (Figs. 1F, I, L, and O). Counting of α-synuclein-positive LB indicated that 80–90% of the cortical LB ($n = 300$, pooled from three DLB cases) were also positive for ROC1 and Cul-1. A similar staining pattern was also observed in LB isolated from the substantia nigra (SN) of PD (data not shown).

Localization of pIκBα, ROC1, and Cul-1 in cytoplasmic inclusions of SH-SY5Y cells

As a model for the formation of cytoplasmic inclusions, we used SH-SY5Y cell lines treated with MG132 [26]. Localization of pIκBα, ROC1, and Cul-1 was investigated after the addition of 10 μM MG132 for 24 h in differentiated SH-SY5Y cells. Proteasomal dysfunction caused typical cytoplasmic inclusions that were stained with anti-ubiquitin (Ub) antibodies, and interestingly many, if not all, ubiquitinated inclusions were also positive for pIκBα, ROC1, and Cul-1 (Figs. 2A–C). Under normal conditions without MG132, the cells displayed low-level cytoplasmic staining for the indicated proteins (data not shown). Although we examined the effect of TNF-α on the formation of the inclusions, no inclusions that contained ubiquitin and pIκBα were observed after treatment with TNF-α alone. In addition, the effect of simultaneous treatment with TNF-α and MG132 was not significantly different from the results of MG132 treatment alone (data not shown).

We next examined whether these cells also contained α-synuclein in such cytoplasmic ubiquitinated inclusions. Following proteasomal inhibition with 10 μM MG132, some of the ubiquitinated cytoplasmic inclusions also exhibited α-synuclein immunoreactivity (Fig. 2D). Moreover, we examined whether pIκBα and components of the SCF complex colocalize with α-synuclein in the presence of 10 μM MG132. The α-synuclein-positive inclusions were also immunoreactive for pIκBα ROC1 and Cul-1 following treatment with 10 μM MG132 (Figs. 2E–G). The proportion of cells treated with 10 μM MG132 that contained aggregates immunoreactive for both pIκBα and α-synuclein was 7.98 ± 1.14%. In contrast, the proportion of 10 μM MG132-treated cells containing inclusions positive for both pIκBα and ubiquitin was 23.19 ± 3.84%, suggesting the relative low frequency of α-synuclein/pIκBα-containing inclusions (see Fig. 5B). Inclusions containing only ubiquitin, α-synuclein, or pIκBα were also noted, and their size was also comparatively heterogeneous (data not shown).

Inhibition of proteasomes increases phosphorylated IκBα level in SH-SY5Y cells

We examined the migration pattern of endogenous ubiquitin or pIκBα by SDS–PAGE in differentiated SH-SY5Y cells following proteasomal inhibition with MG132 and/or TNF-α for 24 h. Cells were treated as indicated in Fig. 3, and then the resulting cell extracts were separated into detergent-soluble and detergent-insoluble fractions. Treatment with 10 μM MG132 resulted in accumulation of high-molecular weight ubiquitin–protein conjugates particularly within the insoluble fractions but not in TNF-α alone and control

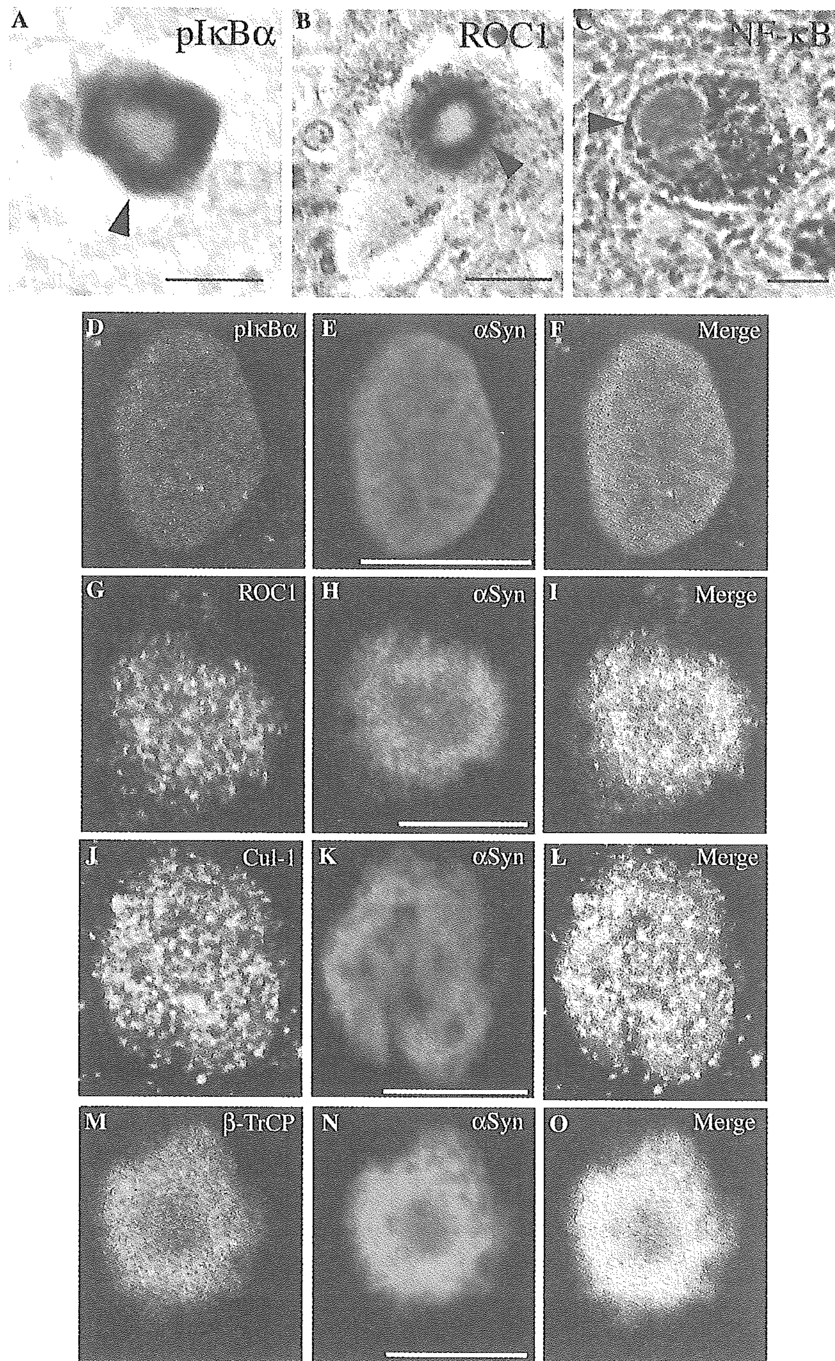

Fig. 1. Identification of phosphorylated IκBα and components of $SCF^{\beta\text{-}TrCP}$ in Lewy bodies. (Upper panel) Paraffin sections of autopsied human brain samples with PD were immunostained with antibodies against pIκBα (A), ROC1 (B), and NF-κB p65 (C). Lewy bodies are marked by arrowheads. Scale bars = 20 μm (A–C). (Lower panel) Colocalization of α-synuclein (αSyn), pIκBα, and components of $SCF^{\beta\text{-}TrCP}$ in isolated LB from DLB (Dementia with LB) cases. LB were identified by α-synuclein staining. Each preparation was doubly stained with sheep anti-α-synuclein (E, H, K, and N) and various antibodies against pIκBα (D), ROC1 (G), Cul-1 (J), and β-TrCP (M), and analyzed with a laser-scanning confocal microscope. Panels (F, I, L, and O) at right correspond to merged images; yellow-colored structures indicate colocalization. Scale bars = 10 μm (D–O).

cells (Fig. 3A). Unexpectedly, the effect of TNF-α was very weak in SH-SY5Y cells, because no massive reduction of IκBα was observed upon treatment with TNF-α for 1, 12, or 24 h (Fig. 3C and data not shown). This finding was in marked contrast to the almost complete disappearance of IκBα in HeLa cells treated with 20 ng/ml TNF-α within 1 h (data not shown). However, TNF-α significantly increased the pIκBα level (Fig. 3B), indicating the existence of TNF-α response to a lesser extent in SH-SY5Y cells. It is of note that MG132 alone

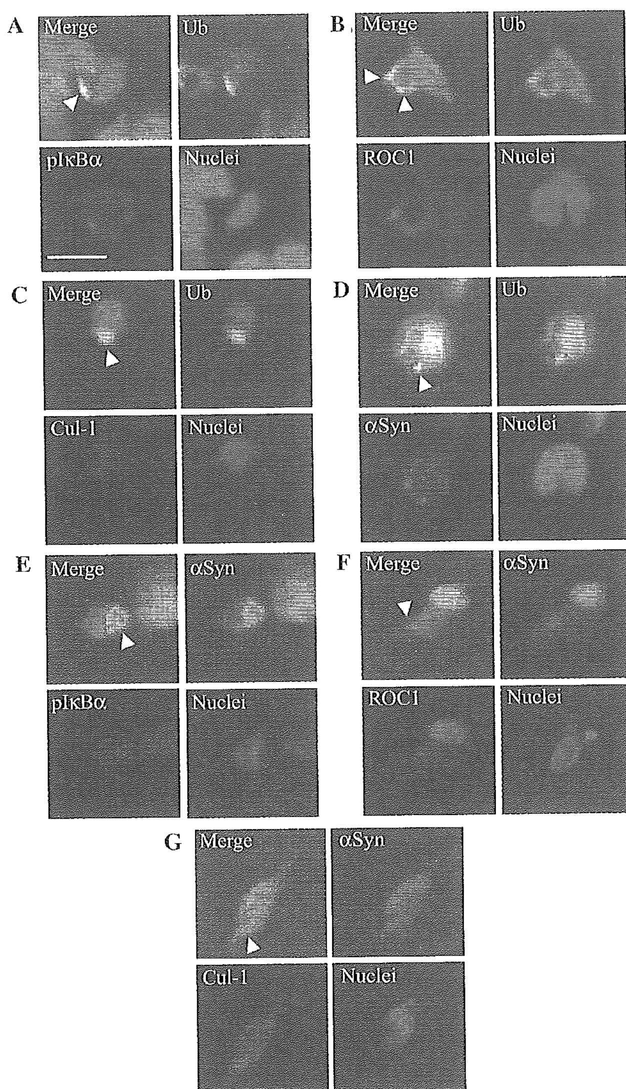

Fig. 2. Proteasomal inhibition leads to formation of pIκBα-positive cytoplasmic inclusions in SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with 10 μM MG132 for 24 h, fixed and then double-stained with various combinations of antibodies as indicated. (A–D) Cytoplasmic inclusions positive for ubiquitin (Ub) were co-stained for pIκBα (A), ROC1 (B), Cul-1 (C), and α-synuclein (D). Arrowheads indicate the inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. (E–G) α-Synuclein (αSyn)-positive cytoplasmic inclusions were co-stained for pIκBα (E), ROC1 (F), and Cul-1 (G). Regions of overlap between α-synuclein (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. Scale bar = 10 μm.

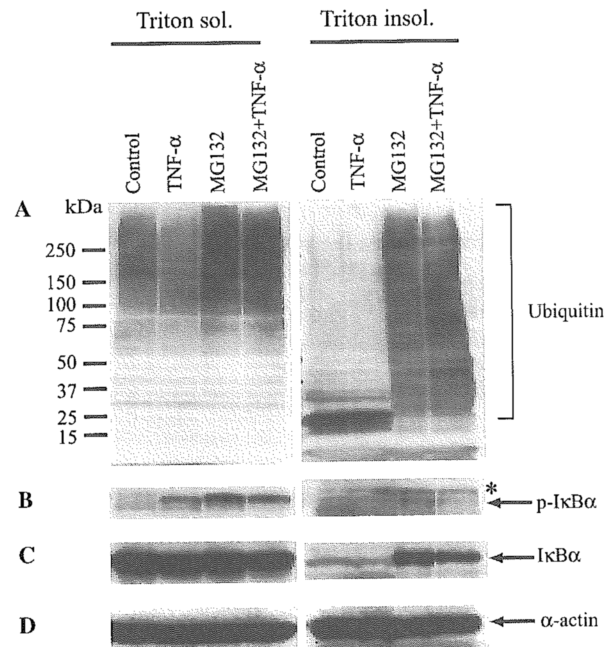

Fig. 3. Inhibition of the proteasome results in accumulation of pIκBα within the detergent-insoluble fraction of SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 and/or 20 ng/ml TNF-α, and the cell lysates were processed for Western blotting, as described in Materials and methods. The protein was blotted onto PVDF membranes and probed with antibodies against ubiquitin (A), pIκBα (B), and IκBα (C). Note that anti-IκBα antibody reacted both phosphorylated and unphosphorylated forms. α-Actin served as a loading control (D). Asterisk indicates a non-specific band.

increased the pIκBα level in the cells (Fig. 3B), although additive effects of TNF-α and MG132 were not observed for phosphorylation of IκBα. Intriguingly, when detergent-soluble and -insoluble fractions were immunoblotted with anti-pIκBα or anti-IκBα antibody, both proteins were clearly detected in the detergent-insoluble fraction after treatment with 10 μM MG132 but not in TNF-α alone and control cells (Figs. 3B and C). In addition, simultaneous treatment with TNF-α and MG132 had no significant effects in comparison with MG132 treatment alone. Thus, it is clear that IκBα, perhaps its phosphorylated form, is incorporated into the detergent-insoluble fraction under the conditions of proteasome inhibition.

The NBD peptide inhibits pIκBα entry into cytoplasmic ubiquitin-positive inclusions

The presence of pIκBα in LB of autopsied brains of PD patients and ubiquitinated inclusions in SH-SY5Y in the present study led us to examine whether inhibition of IKK, which phosphorylates IκBα, alters the processes of inclusion formation and cell death. First, we determined the optimal concentration of the cell-permeable NBD peptide, which is known to block the activity of IKK. To study the effect of NBD, SH-SY5Y cells were pre-treated with various concentrations of wild-type NBD for 3 h and then stimulated by 20 ng/ml TNF-α. In the present study, we used 40 μM NBD as the optimal concentration to block phosphorylation of IκBα. We also examined the effect of high concentrations of the NBD peptide (about 1000 μM), as described previously [21], but peptide toxicity was observed in our cell lines.

We next treated the cells with MG132 in the presence or absence of NBD peptide and then performed double

staining using antibodies for pIκBα, IκBα, and ubiquitin. Ubiquitinated inclusions containing pIκBα were identified in cells treated with MG132 alone or with MG132 in the presence of mutant NBD lacking inhibitory activity for IKK. On the other hand, while ubiquitinated inclusions were observed in cells treated with MG132 in the presence of wild-type NBD, only a few cells contained ubiquitinated inclusions positive for pIκBα (Fig. 4A). In addition, the use of an antibody for IκBα in the presence of wild-type NBD was also associated with reduced number of cells with ubiquitinated inclusions positive for IκBα, compared with those treated with MG132 or MG132 in the presence of mutant NBD (Fig. 4B), indicating that phosphorylation of IκBα may be required for its incorporation into cytoplasmic inclusions generated by proteasome inhibition.

We then counted the number of cells with aggregated immunoreactivity for both ubiquitin and pIκBα antibodies under basal condition and following treatment with 10 μM MG132 with or without NBD peptide. It is worth noting that whereas approximately 50% of total cells contained ubiquitin-positive inclusions, pIκBα-positive inclusions were below 25% (Figs. 5A and B), suggesting that pIκBα is not incorporated into all inclusions. Wild-type NBD significantly decreased the number of cells with ubiquitinated inclusions (Fig. 5A, $p < 0.05$), and cells with cytoplasmic inclusions positive for pIκBα and ubiquitin, compared with cells treated with MG132 alone (Fig. 5B, $p < 0.001$). In comparison, mutant NBD did not show the same effects on phosphorylation of IκBα as wild-type NBD. Finally, we examined the toxicity of 10 μM MG132 on these cell lines. Treatment with 10 μM MG132 reduced cell viability to $37.84 \pm 1.46\%$. In contrast, wild-type NBD did not influence cell viability under proteasomal inhibition (Fig. 5C).

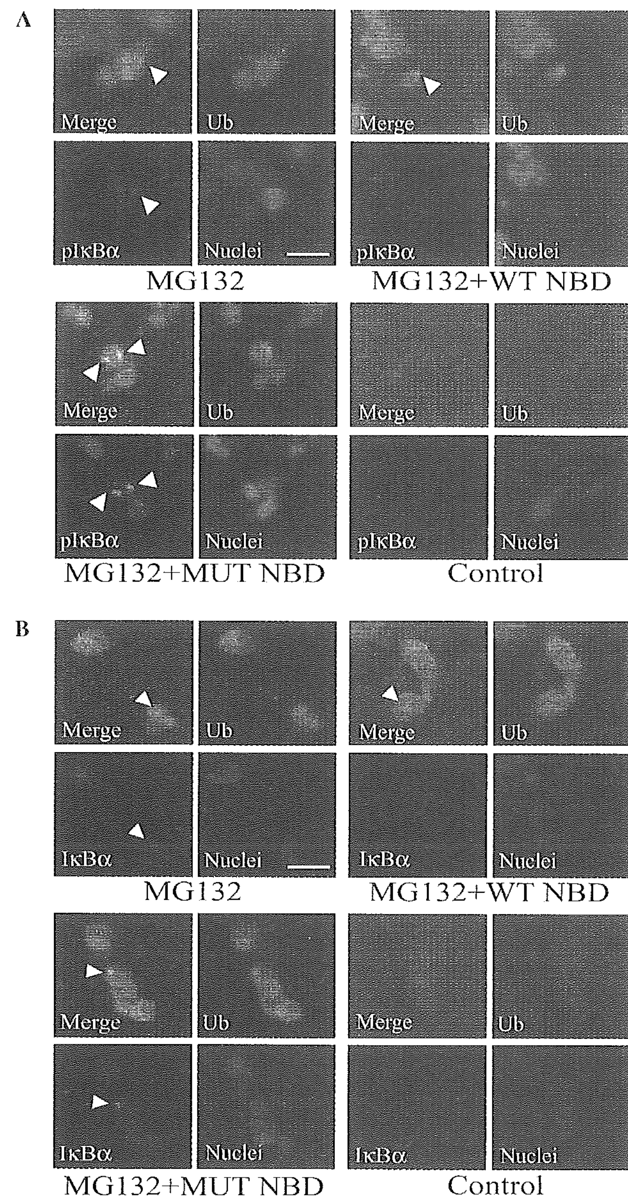

Fig. 4. Wild-type NBD decreases pIκBα level within the ubiquitinated inclusions in SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 alone, or with 10 μM MG132 in the presence of 40 μM of either wild-type (WT NBD) or mutant NBD peptide (MUT NBD) as indicated in Materials and methods. Cells with ubiquitinated inclusions were co-stained with pIκBα (A) and IκBα (B). Arrowheads indicate the ubiquitinated inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) are shown in yellow color. Scale bar = 20 μm.

Discussion

The appearance of LB in SN is a prominent feature in PD, but the pathogenic role of such inclusions remains elusive. In this study, we identified novel components including pIκBα and components of SCF$^{\beta\text{-TrCP}}$ ligase in LB. To date, several studies have reported that the UPP-related proteins (such as ubiquitin, the 20S proteasome subunit, and HSP70) are localized in LB of PD [27,28]. These findings indicate that there appears to be an important correlation between some pathological alteration in UPP and the formation of LB in PD. In this regard, the pathogenic nature of proteasomal dysfunction has been studied in experimental models using a proteasome inhibitor. It has been demonstrated that inhibition of proteasomal function induces the formation of cytoplasmic inclusions immunoreactive for ubiquitin and α-synuclein in PC12 cells and mesencephalic cultures [20,29]. These observations suggest that proteasomal dysfunction is associated with the development of cytoplasmic inclusions that may have features similar to those of LB, in terms of containing two proteins; i.e., α-synuclein and ubiquitin, described as the major components of LB [2].

pIκBα and SCF ligase are also involved in UPP-related proteins, and these molecules have not been adequately studied in PD. Therefore, to explore how these

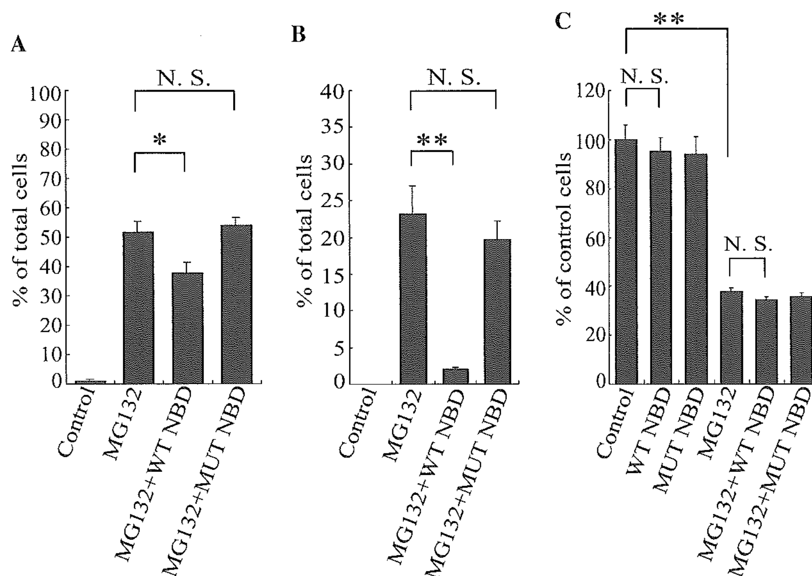

Fig. 5. Effects of NBD on formation of pIκBα-containing inclusions and cell death caused by proteasomal inhibition. After the cells were treated as explained in Fig. 4, the proportions of cells with cytoplasmic inclusions were determined. (A) The proportion of cells with ubiquitin-positive inclusions was calculated relative to total cells. (B) The proportion of cells with ubiquitinated inclusions containing pIκBα was calculated relative to total cells. In each experiment, 10 fields of 50 cells were counted. Similar effects of NBD were seen in two or more independent experiments. Data are means ± SEM. *$p < 0.05$; **$p < 0.001$ for differences between cell lines (Tukey's multiple t test). NS, not significant. (C) Cell viability was assessed as described in Materials and methods, and is expressed as the percentage of untreated cells. Similar results were seen in three independent experiments. Values are means ± SEM, each $n = 8$. **$p < 0.001$ for differences between cell lines (Tukey's multiple t test).

molecules are present in LB would be important in considering the process of LB formation. In our cell culture model, inhibition of normal proteasomal function by MG132 also induced the formation of ubiquitinated cytoplasmic inclusions containing α-synuclein, and this finding is consistent with previous reports [20,29], as described above. Intriguingly, our results showed that these inclusions were positive for pIκBα and some components of its ligase that are found in the LB. These findings suggest that the existence of pIκBα in LB is more likely and proteasomal dysfunction is an important factor in the formation of cytoplasmic inclusions.

Using SDS–PAGE analysis of detergent-soluble and -insoluble fractions, we found high-molecular weight ubiquitinated proteins particularly in the detergent-insoluble fraction, and pIκBα in the insoluble fraction following proteasomal inhibition with MG132. In contrast, after incubation with TNF-α alone, neither high-molecular weight ubiquitinated bands nor pIκBα was detected in the insoluble fractions, and cytoplasmic inclusions containing ubiquitin and pIκBα were not observed. These findings suggest that phosphorylation of IκBα alone is insufficient for the formation of cytoplasmic inclusions, and there appears to be a strong causal link between the accumulation of poorly degraded proteins, resulting from proteasomal dysfunction, and the formation of cytoplasmic inclusions.

We also showed that the presence of pIκBα in the ubiquitinated inclusions was markedly inhibited by a specific IKK inhibitor, under the conditions of MG132 treatment. This finding also supports the above-mentioned data that pIκBα is involved in the cytoplasmic inclusions resulting from proteasomal inhibition in our SH-SY5Y cells. In addition, this finding provides us a further possibility. In some neurodegenerative disorders, the ubiquitin-positive inclusions are considered to involve the ubiquitin–protein conjugates [28,30]. However, it is not clear which types of proteins are directly polyubiquitinated in LB. IκBα is phosphorylated by IKK, and pIκBα is polyubiquitinated by the $SCF^{\beta\text{-TrCP}}$, then degraded by the 26S proteasome. Thus, it is conceivable that once phosphorylation of IκBα is inhibited, neither polyubiquitination after its phosphorylation nor accumulation of IκBα into inclusion bodies is observed. We demonstrated that wild-type NBD peptide reduced the proportion of not only ubiquitin-positive inclusions, but also ubiquitinated inclusions containing pIκBα. Based on our finding, it is possible that the polyubiquitination of pIκBα resulting from proteasomal dysfunction triggers its entry into ubiquitinated cytoplasmic inclusions.

It is still not clear whether LB are cytoprotective or cytotoxic for neurons in the SN of PD. Recent studies suggest that the formation of protein aggregates or intracellular inclusions may be beneficial for cell survival rather than enhance cell death [31,32]. In the present study, exposure to MG132 alone or MG132 in the presence of wild-type NBD peptide did not alter cell viability whereas the same conditions decreased the ubiquitinated cytoplasmic inclusions. This finding at least supports the