A Product of the Human Gene Adjacent to parkin Is a Component of Lewy Bodies and Suppresses Pael Receptor-induced Cell Death*

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Parkin, a RING-type ubiquitin ligase, is the product of the gene responsible for autosomal recessive juvenile parkinsonism. A reverse strand gene located upstream of the parkin gene in the human genome has been identified. The gene product, termed Glup/PACRG, forms a large molecular chaperone complex containing heat shock proteins 70 and 90 and chaperonin components. Glup suppressed cell death induced by accumulation of unfolded Pael receptor (Pael-R), a substrate of Parkin. On the other hand, Glup facilitated the formation of inclusions consisting of Pael-R, molecular chaperones, protein degradation molecules, and Glup itself, when proteasome is inhibited. Glup knockdown attenuated the formation of Pael-R inclusions, which resulted in the promotion of cell death with extensive vacuolization. Moreover, Glup turned out to be a component of Lewy bodies in Parkinson’s disease cases. These data suggest that Glup may play an important role in the formation of Lewy bodies and protection of dopaminergic neurons against Parkinson’s disease.

Parkinson’s disease (PD)† is a movement disorder characterized pathologically as a progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Most cases of PD are sporadic, and the etiology of common PD remains unknown. However, recent identification of gene mutations in familial cases of PD has advanced our understanding of the molecular mechanisms behind the neurodegeneration associated with this disease.

Two rare missense mutations in the α-synuclein (α-SYN) gene (A53T and A30P) have been found to cause autosomal dominant familial PD (1, 2). Although the physiological function of α-SYN is still unclear, there is evidence that even wild-type α-SYN is a major component of Lewy bodies (LBs) and that its overexpression in animal models causes neurodegeneration, thus suggesting that α-SYN might be important in the etiology of PD (3). LBs are neuronal inclusions that are frequently found in various regions, including the substantia nigra, of the brains of PD patients. This finding strongly suggests the involvement of abnormal protein turnover in PD.

Autosomal recessive juvenile Parkinsonism (AR-JP) is a major cause of juvenile PD and results from mutations in the parkin gene (4). In AR-JP patients, loss of dopaminergic neurons and subsequent parkinsonian symptoms basically develop without LB pathology (5). parkin is one of the largest genes of the human genome (1.4 Mb), composed of 12 exons encoding a 465-amino acid protein with a molecular mass of 52 kDa (4, 6). Parkin protein is a RING finger-type ubiquitin ligase, and AR-JP-linked Parkin mutants are defective in ubiquitin ligase activity (7–9). The accumulation of unfolded Pael-R, a substrate for Parkin recently identified in our laboratory, causes unfolded protein stress, which results in cell death. Parkin eliminates unfolded Pael-R, in cooperation with the molecular chaperone Hsp70 and a U box protein, known as CHIP. Other findings in transgenic α-SYN toxicity have demonstrated that molecular chaperones attenuate unfolded protein toxicity, without suppressing the formation of inclusion bodies (10). Thus, protein degradation and the molecular chaperone system appear to be closely associated with the pathogenesis and prevention of PD.

During structural analysis of the parkin gene, another gene was found to be located only 200 bp upstream of the parkin gene (11, 12). A high degree of conservation was observed among the exons and introns of these two genes in mice and humans. Here we report that the gene located upstream of parkin (designated as Glup) is one component of LBs and suppresses unfolded Pael-R-mediated neural death, promoting formation of Pael-R inclusion bodies.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Proteins—Expression plasmids for human Parkin, Hsp70, CHIP, p38, XIAP, c-IAP1 and -2 and Pael-R are described elsewhere (7, 13–15). Full-length cDNAs of human and mouse Glup and human β-actin were cloned by reverse transcription and PCR from a human brain cDNA library or first strand cDNA from Neuro2A cells and subcloned into the mammalian expression vectors pcDNA3, with or without various tags (Invitrogen). For RNA experiments, the sequences of human Glup, 5′-AAGATCTCAGTGTCTCCAC-3′ (for clone h4), and murine Glup, 5′-AAGATCTTACCTGACATCCTCG3′ (for clone m5), were inserted into pSUPER vector to generate short hairpin RNA. Anti-Glup Ab was raised against GST-Glup after removal of the GST

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†The abbreviations used are: PD, Parkinson’s disease; PACRG, PAR2 co-regulated gene; AR-JP, autosomal recessive juvenile parkinsonism; Pael-R, Pael receptor; Hsp, heat shock protein; LB, Lewy body; α-SYN, α-synuclein; CHIP, C terminus of the Hsc-interacting protein; glup, gene located upstream of parkin; Hsc, heat shock cognate protein; Hop, Hsp70/Hsp90 organizing protein; TCP1, t-complex polypeptide 1; HA, hemagglutinin; Ab, antibody; ER, endoplasmic reticulum; VCP, valosine-containing protein; EGFP, enhanced green fluorescence protein; XIAP, X-linked inhibitor of apoptosis; c-IAP, cellular inhibitor of apoptosis; RNAi, RNA interference; GST, glutathione S-transferase.

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moiety. For the most part, full-length GST-Glup is difficult to express in bacteria, and it is therefore expressed as an N-terminal truncated form with a GST moiety. The Ab is affinity-purified by the antigen. Anti-VCP Ab was generated against recombinant His6-tagged (His6/H11002) VCP expressed in bacteria. Anti-TCP1/H9251 (23c and 84a) and anti-TCP1/H9252 (CTA-200) Abs were from StressGen. Anti-TCP1/H9252 (C-17), anti-Hsp90 (H-114), and anti-Hsp70 (K-20 and W-24) Abs were purchased from Santa Cruz Biotechnology. Anti-FLAG (M2) and anti-HA (3F10) and anti-Hop (28) Abs were purchased from Sigma. Anti-Parkin, anti-Pael-R, anti-CHIP, and anti-Hdj2 Ab are described elsewhere (7, 13, 15). Anti-Myc (9E10) was prepared in our laboratory. For immunoabsorption experiments, FLAG-Glup, FLAG-Parkin, Pael-R-FLAG, and TCP1/H9252-Myc purified from cultured cells as well as recombinant His-Hsp70, His-VCP, and His-Hdj2 from bacteria were used for the corresponding Abs.

Cell Culture, Transfection, and Cell Death Assays—Human embryonic kidney 293T and neuroblastoma SH-SY5Y cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine fetal calf serum. Human embryonic kidney 293 cells transfected with pcDNA3 bearing FLAG-Glup, FLAG-Parkin, or Pael-R-FLAG were selected with 1 mg/ml G418. The cells stably expressing these proteins were isolated as 293/FLAG-Glup, 293/FLAG-Parkin, or 293/FLAG-Pael-R cells, respectively. Primary midbrain cultures were prepared from E12.5 mouse embryos and were maintained with Neurobasal Medium supplemented with B27 (Invitrogen). Transfection and cell death assays using PC12 cells expressing Pael-R with EGFP (P1 cell) were carried out as described previously (15). The amounts of total DNA for transient transfection were equally adjusted.

Immunoprecipitation, Western Blot, and Fractionation—Immunoprecipitation, Western blot, and detergent-soluble/insoluble fractionation were performed as described previously (13). Immunoabsorption experiments, FLAG-Glup, FLAG-Parkin, Pael-R-FLAG, and TCP1/H9252-Myc purified from cultured cells were used for the corresponding Abs.

RESULTS

Glup Forms a Complex with Chaperones—Because there have been recent reports that Glup/PARC is co-regulated...
with Parkin by the shared promoter, we expected Glup to be functionally related to Parkin. To elucidate its physiological function in the cell, we biochemically isolated Glup-binding proteins (Fig. 1A). The binding proteins included Hsp70, heat shock cognate protein (Hsc) 70, Hsp90, a co-chaperone Hop, components of mammalian chaperonin, and known substrates of these chaperones tubulins and actin (16–18). Among them, Hsc/Hsp70 and actin were also detected with the FLAG-Parkin immunoprecipitate. We detected seven of eight components of chaperonin in the biochemically isolated Glup complex and confirmed by immunoprecipitation/Western blot analysis that the last component TCP1β is located in the Glup complex (Fig. 1B). The components of chaperonin, TCP1α and -β, interacted with Glup with an N-terminal FLAG tag (FLAG-Glup) but only weakly with FLAG-Parkin, whereas Hsp70 bound to FLAG-Glup as well as FLAG-Parkin and Pael-R with a C-terminal FLAG-tag (Pael-R-FLAG) (Fig. 1B). The interaction of Hsp90 with Glup was more specific than other proteins (Fig. 1B). Gel filtration analysis revealed that Glup could form a large complex with Hsp90, Hsp70, Hop, TCP1α (Fig. 1C), TCP1β, and TCP1θ (data not shown).

Because Glup binds to chaperonin much more strongly than Parkin and Pael-R and because the molecular size of Glup is suitable for the pore of chaperonin, it is possible that Glup is one of the substrates for chaperonin. To examine whether Glup is a substrate of chaperonin, we co-immunoprecipitated FLAG-Glup with TCP1β or TCP1α following synthesis of FLAG-Glup in the reticulocyte lysate (Fig. 2A). The amount of co-immunoprecipitated Glup was essentially constant up to 40 min after Glup synthesis was terminated, during the time in which the known chaperonin substrate β-actin was rapidly released from TCP1α (19). We next pulse-labeled 293 cells stably expressing FLAG-Glup (293/FLAG-Glup cells) with [35S]methionine/cysteine and then chased the amount of co-immunoprecipitated FLAG-Glup or β-actin with TCP1α (Fig. 2B). The pulse-chase experiment also showed that Glup, but not β-actin, constitutively associates with a component of chaperonin. Finally, we investigated the effects of ATP, GTP, or these derivatives on the binding of Glup to a chaperonin component (Fig. 2C). The affinity between Glup and TCP1β was completely unaffected by these reagents. These results strongly suggest that Glup is not a substrate of chaperonin.

Glup Binds to Parkin and Pael-R—Glup forms a complex with molecular chaperones such as Hsp70, and because Parkin also binds to Hsp70, we expected that Glup would also bind to Parkin, either directly or via Hsp70. Transient transfection and subsequent immunoprecipitation experiments revealed that hemagglutinin (HA)-tagged Glup specifically binds to FLAG-Parkin (Fig. 3A). Next, we generated an anti-Glup antibody (Ab), and we confirmed that the Ab recognized ~28-kDa proteins, which are expressed abundantly in the cortex, the brainstem, and the spinal cord and weakly in the cerebellum (Fig. 3, B and C, and data not shown). With the anti-Glup Ab, we detected anti-Glup-positive signals in the anti-Parkin immunoprecipitate, both from cultured cells and brain tissue (Fig. 3C). The amount of associated Glup with Parkin was increased in the Parkin immunoprecipitate following transient Hsp70 overexpression (Fig. 3D). Although transient TCP1β overexpression dramatically promotes TCP1β incorporation into the Parkin immunoprecipitate, its ability to recruit Glup into the Parkin complex is less than that of Hsp70 (Fig. 3E). Further association analysis in Hsp70 co-expression with Glup or Parkin and treatment of proteasome inhibitors (MG-132 and lactacystin) were carried out using 293 cells stably expressing FLAG-Parkin or FLAG-Glup. Hsp70 overexpression, inhibition of proteasome activity, or a combination of these led to a mild increase in Hsp70 recruitment into the Parkin or Glup immunoprecipitates (Fig. 3E, lanes 3–6 and 15–18 in the 3rd row) and led to an increase in Parkin-Glup binding (Fig. 3E, lanes 3–6 and 15–18 in the 2nd row). As reported previously, Parkin-immunoprecipitated complex contained a U box protein CHIP and an endoplasmic reticulum (ER) resident Hsp40 Hdj-2 (Fig. 3E, the 4th and 5th row). CHIP was constitutively present in the Glup immunoprecipitate even in the absence of Parkin (Fig. 3E, 4th row). However, Hsp70 overexpression or proteasome inhibition only slightly promoted the association of CHIP with Glup. In contrast, Hdj-2 was recruited to Glup only during proteasome inhibition with MG-132 or lactacystin (Fig. 3E, lanes 15, 16, and 18 in the 5th row). VCP, which is an AAA-ATPase family molecule involved in the organization of cellular membranes and ER-associated degradation, was detected in both Parkin and Glup immunoprecipitates only upon proteasome inhibition (Fig. 3E, the 6th row). Binding of VCP to Glup was stronger than to Parkin. Glup binding-chaperones such as
Hsp90, TCP1α, and TCP1β seemed to be constantly associated with Glup and very weakly with Parkin during these treatments (Fig. 3E, 7th to 9th rows).

We next examined whether Pael-R was able to associate with Glup in the presence or absence of Hsp70 or TCP1β overexpression (Fig. 4A). Unlike Parkin, Pael-R was hardly associated with TCP1β, as shown in Fig. 1B. Interaction between Glup and Pael-R, similar to that between Glup and Parkin, was promoted by Hsp70, but not by TCP1β, and was dramatically strengthened by the presence of the proteasome inhibitor MG-132 (Fig. 4B).

Thus, overexpression of Hsp70 and proteasome inhibition promoted interaction of Parkin and Pael-R with Glup probably because the up-regulated Hsp70 facilitates direct binding of Parkin and Pael-R to Glup and because some of the Hsp70 bound to Parkin and Pael-R will recruit Glup. In contrast, the marked increase in Glup binding to Pael-R after proteasome inhibition did not appear only due to Hsp70 up-regulation.

Glup Is a Component of Lewy Bodies—Immunoprecipitation analysis showed that inhibition of proteasome activity effectively led to recruitment of Glup to Parkin and particularly to Pael-R. To explore the mechanism underlying the effect of proteasome inhibition, immunocytochemical analysis was performed in the presence or absence of a proteasome inhibitor. Glup is normally associated with vesicles and sometimes shows filamentous structures within the cytosol and neurites of primary cultured neurons (Fig. 5A) and neuroblastoma SH-SY5Y cells (data not shown). We have reported that Pael-R forms aggresome-like inclusions in the perinuclear region of cells overexpressing Pael-R in the presence of MG-132 (13). Under these conditions, when Glup was co-expressed together with Pael-R, Glup was co-localized with the perinuclear inclusion of Pael-R (Fig. 5B). The cellular localization of Glup showed a basically punctate pattern in the cells and largely overlapped with TCP1β and partially overlapped with Hsp70, Parkin, and CHIP under physiological conditions (Fig. 5, C–E and H, left).
Figure 4. The association of Glup with Pael-R. A, 293 cells stably expressing Pael-R-FLAG (293/FLAG-Pael-R cells, 1.7 x 10^6 each) were transfected, followed by immunoprecipitation with anti-FLAG Ab (FLAG-IP) as in Fig. 3D. Western blotting was carried out with Abs specific to the indicated proteins. B, 293/FLAG-Pael-R (1.7 x 10^6 each) were transfected with Glup combined with an empty vector or His-Hsp70 and were treated with or without MG-132. Immunoprecipitation and subsequent Western blotting were carried out as in A. Western blotting of soluble lysate (Total) is also shown.

Although the binding of Hdj-2 and VCP to Glup was not detected in the immunoprecipitation analysis, partial co-localization of these molecules was observed, suggesting that VCP and Hdj-2 transiently associated with Glup in cells (Fig. 5, F and G, left columns). On the other hand, when cells expressing Pael-R were treated with MG-132, they formed Pael-R-positive inclusions, the cores of which contained Parkin, Hsp70, CHIP and Hdj-2 (Fig. 5, C–F, middle and right columns). In contrast, VCP accumulated in the periphery of the inclusions rather than in the central regions (Fig. 5G, middle and right columns). TCP1β was either not recruited into the inclusion (Fig. 5H, upper part in the middle and right columns) or overlapped more scarcely (Fig. 5H, lower part in the middle and right columns). The specificity of the antibodies used in the immunocytochemical experiments was confirmed by immunoblotting with the antigen (Fig. 5I). These results seemed to explain the results of the immunoprecipitation assay shown in Fig. 4E.

The presence of Glup in the Pael-R-positive inclusions led us to examine whether Glup is a component of LBs (Fig. 6). Anti-Glup Ab strongly recognized the LBs in cases with sporadic PD and dementia with LBs (Fig. 6, A–C). In contrast, incubation with Ab solution preabsorbed against FLAG-Glup purified from 293/FLAG-Glup cells (preabsorbed) did not show any signals in the brain sections, indicating that the anti-Glup Ab specifically recognizes Glup protein (Fig. 6D).

Glup Forms Inclusions of High Molecular Weight in the Presence of Proteasome Inhibitors—Previous reports (10, 20–22) have demonstrated that Hsp70 and Parkin are components of LB or cellular inclusions. Therefore, Hsp70 or Parkin may recruit Glup into these inclusions. To examine this possibility, FLAG-Glup, immunoprecipitated from 293/FLAG-Glup cells treated with or without MG-132, was subjected to a gel filtration chromatography (Fig. 7A). The result revealed that part of Glup was >1 MDa of the high molecular mass in the presence of MG-132 (Fig. 7A, fractions 1–8). The amounts of co-immunoprecipitated Parkin and Hsp70 were increased in the presence of MG-132, as shown in Fig. 3E. Moreover, MG-132 treatment also promoted the recruitment of Parkin and Hsp70, but not TCP1α or -β, to the high molecular weight fractions. Consistent with the gel filtration experiment, an immunocytochemical analysis showed that the subcellular localization of both overexpressed and endogenous Glup were dramatically changed by MG-132 treatment, which led Glup into γ-tubulin-positive large inclusions, so-called aggresomes (Fig. 7C and F compared with B, and data not shown). In contrast, Hsp70 and TCP1β did not show any cytoplasmic aggregates in the presence of MG-132, even when overexpressed in SH-SY5Y, Neuro2A, and 293/FLAG-Glup cells (Fig. 7E, F and H, and data not shown). A previous study reported that overexpressed Parkin forms aggresomes upon proteasome inhibition in COS-7 cells (23). We also observed that both overexpressed and endogenous Parkin were recruited into γ-tubulin-positive inclusions in MG-132-treated SH-SY5Y (Fig. 7, D and G) and Neuro2A cells (data not shown). However, the aggresome-forming efficiencies of both overexpressed and endogenous Glup were much higher than that of Parkin (Fig. 7H).

Moreover, transient co-expression of Glup with Parkin-Myc or His-Hsp70 in the presence of MG-132 facilitated the aggresome formation of Parkin-Myc or His-Hsp70 in SH-SY5Y (Fig. 7I) and Neuro2A cells (data not shown). Taken together, our results indicated that Glup rather recruits Parkin or Hsp70 into cytoplasmic aggregates than otherwise, suggesting that Glup may be one of cytoplasmic aggregate-promoting factors.

Glup Suppresses Pael-R-induced Cell Death, Promoting Cytoplasmic Inclusions—We predicted that Glup might protect neuronal cells against cell death induced by unfolded Pael-R protein stress, because Glup forms a large complex containing molecular chaperones. We first examined the effect of Glup on Pael-R overexpression in SH-SY5Y cells (Fig. 8A). As reported previously, co-expression of Parkin and Pael-R suppressed the formation of Triton X-100-insoluble Pael-R–immunoreactive material, which represents unfolded and insolubilized Pael-R, whereas inhibition of proteasomal activity through treatment with MG-132 enhanced the accumulation of Pael-R–immunoreactive material within the Triton X-100-insoluble fraction (13). Co-expression of Glup and Pael-R had a slight but significant inhibitory effect on the accumulation of insoluble Pael-R, and the addition of Parkin expression had an additive inhibitory effect. Under these conditions, we performed a cell death assay by Pael-R overexpression (Fig. 8B). Transfection of Pael-R led to the death of the transfected cells, and treatment with MG-132 during Pael-R expression caused a dramatic increase in cell death. Co-expression of Parkin suppressed cell death by ~50%. Transfection of Glup also suppressed cell death to the same extent, although Glup only weakly inhibited the accumulation of unfolded Pael-R. Co-expression of Parkin and Glup with Pael-R effectively suppressed cell death by ~70%.

PC12 cells expressing Pael-R-EGFP in an inducible manner undergo cell death when Pael-R-EGFP protein expression is induced (15). Thus, we performed a cell survival assay by transient co-transfection of these cells with various cDNAs, including some encoding anti-apoptotic factors (Fig. 8C). The ER chaperone, BiP, offers significant protection against Pael-R-EGFP-induced cell death. Parkin, Hsp70, and CHIP also...
FIG. 5. Glup is recruited into Paell-R inclusions. A, primary cultured neurons from the mouse midbrain were stained with anti-Glup Ab (green) and counterstained with 4',6-diamidino-2-phenylindole (blue) to visualize the nucleus. High power magnification of the axonal structures of the left picture is shown on the right. All bars represent 10 μm. B, neuroblastoma SH-SY5Y cells transfected with Venus (an improved version of yellow fluorescent protein)-fusioned Glup and monomeric red fluorescence protein-fusioned Paell-R were treated with 10 μM MG-132 for 12 h after 20 h post-transfection (30, 31). Arrowheads indicate a perinuclear inclusion. C and E–G, SH-SY5Y cells transfected with Parkin, HA-Glup, and Paell-R were treated with or without MG-132 as in B. The cells were stained with anti-HA or Abs specific to the indicated proteins 36 h after transfection. Arrowheads indicate a Paell-R-immunopositive inclusion. D and H, SH-SY5Y cells transfected with Paell-R were treated with or without MG-132 as in B. The cells were stained with Abs specific to the indicated proteins 36 h after transfection. Arrowheads indicate a Paell-R-immunopositive inclusion. I, a, SH-SY5Y cells treated with MG-132 after transfection with Paell-R as in B–H were stained with anti-Paell-R.
suppress cell death following induction of Pael-R-EGFP, as reported previously (15). Moreover, Glup effectively suppressed Pael-R-induced cell death in the present experiment. In contrast, various caspase inhibitory proteins, including XIAP, c-IAP1, c-IAP-2, and p35, did not significantly protect against cell death, suggesting that unfolded Pael-R-induced cell death is not a typical apoptosis process but a caspase-independent process. Similar results were obtained in cell survival assays using Pael-R-transfected differentiated Neuro2A cells (data not shown).

To examine further the function of Glup in cells, we used RNA interference (RNAi). Western blotting showed that human (SH-SY5Y) and mouse (Neuro2A) Glup proteins were specifically reduced after transfection of the plasmids for Glup RNAi (clone h4 for human and clones m4 and m5 for mouse Glup). The efficiencies of these decreases using h4, m4, and m5 were ~85, 95, and 90%, respectively, whereas Parkin levels were not significantly affected (Fig. 9A). Next, we examined the effect of Glup knockdown or overexpression on Pael-R-induced cell death in the presence or absence of MG-132 (Fig. 9B). SH-SY5Y or Neuro2A cells were transfected with Pael-R plasmid together with or without plasmid encoding Glup or Glup RNAi in the presence or absence of MG-132. MG-132 treatment after Glup co-transfection promoted the formation of the Pael-R inclusions during the time in which these cells demonstrated firm adhesion to the culture dish, with the neurites and the integrity of their cytoplasmic membranes being maintained, suggesting that these cells remained healthy even when harboring the inclusions (Fig. 9, B and C). Glup knockdown by RNAi in association with MG-132 led to cell death with abnormal vacuolization of cytoplasm without obvious inclusions (Fig. 9D). The morphology of the cell death induced by Glup knockdown was quite distinct from that induced by Pael-R overexpression, which contained a large perinuclear inclusion in the cell (Fig. 9D, box). These effects were well correlated with the RNAi efficiency of each clone. Moreover, Glup overexpression partially abrogated the effect of Glup RNAi. These observations implied that the formation of cytoplasmic inclusions by Glup ensures the prevention of the cell death induced by unfolded proteins, at least in the short term.

Ab (blue), together with anti-Glup Ab solution preabsorbed with FLAG-Glup purified from 293/FLAG-Glup cells. The Glup signals (green) in both the Pael-R inclusions (arrowheads) and the cytosol almost disappeared. A similar result was obtained by the anti-Pael-R preabsorption (data not shown). b–f, SH-SY5Y cells treated as above were stained with anti-Pael-R (blue) and anti-Glup (green) Abs together with anti-Parkin (b), anti-Hsp70 (c), anti-VCP (d), anti-Hdj-2 (e), or anti-TCP1β (f) Ab solution preabsorbed with the antigens (red). The disappearance of the signals of these Abs after preabsorption indicated the specificity of these Abs.

**FIG. 5—continued**
DISCUSSION

glup (alternatively called HAK005771 or PACRG) has been identified as a gene that lies in close proximity to the parkin gene responsible for juvenile Parkinson’s disease (11, 24). The glup and parkin genes are arranged in a head-to-head orientation with a 200-bp sequence between them. This configuration suggests that the two genes might share a bi-directional promoter. Previous analyses of promoter activity using the reporter genes indicate that these genes are indeed co-regulated at the transcriptional level (12). Unlike Parkin, however, Glup protein expression appears largely restricted to the central nervous system, indicating that glup is also regulated at a post-translational level (data not shown). In the central nervous system, a number of neuronal populations, including dopaminergic neurons, are Glup-immunoreactive in the neuronal fibers as well as in the neuronal bodies. These observations suggest that Glup is important for maintenance of neuronal function.

Although the physiological significance of the observed interaction between Glup and Parkin remains unclear, an increase in the amount of Hsp70 in Parkin complex led to an increase in Glup, suggesting that Hsp70 may mediate the binding between Glup and Parkin. Glup failed to promote the ubiquitin ligase activity of Parkin or the degradation of Pael-R in vitro (data not shown). However, overexpression of Glup was observed to suppress cell death induced by overexpressed Pael-R in various neuronal cell lines. This protective effect of Glup appeared to be similar to that of Hsp70, because overexpression of Glup increased the amount of soluble fraction of Pael-R (see Fig. 4A). Furthermore, the binding property of Glup to TCP1 chaperonin components appears to be stable and strong in the presence of ATP, although the possibility that...
Glup is just a substrate of Hsp70 or Hsp90 cannot be formally excluded. Given that Glup is a positive co-factor of molecular chaperones, it is consistent with the observations that Glup 1) considered dead. The values are means ± S.D. from triplicate samples of three independent experiments. Asterisk, p < 0.05; double asterisk, p < 0.01 versus results obtained after vector transfection.
shown), also increased the total amount of cellular Glup and co-expression, which did not lead to Glup aggregation (data not shown), are growing number of reports showing Lbs sequester protein-folding machineries and molecules involved in the protein degradation. In many experiments using cultured cells, as well as in this study, it is suggested that the disturbance of proteasome activity during overexpression of certain proteins leads to the formation of the unfolded protein inclusion such as aggregates. These cytoplasmic or nuclear inclusions are also reported to sequester molecular chaperones, transcription factor complexes, and ubiquitin-proteasome-related molecules, which might lead to impairment of cellular activity and survival (25, 26). These observations in cultured cells are likely to, at least in part, reflect the formation of Lbs in dopaminergic neurons of PD patients, in which the failure of the ubiquitin-proteasome system is implicated as the primary cause, and a long-term maturation process will be required to convert the amorphous aggregates into Lbs consisting of 10-nm amyloid fibrils (20). Interestingly, the promotion of the formation of Pael-R inclusion by Glup seems to have a positive correlation with the protective function of Glup against cell death by Pael-R overexpression. This observation might support the notion that intermediates of unfolded proteins are more toxic than filamentous Lbs or cytoplasmic inclusions (27). According to our hypothesis, Glup aggregation might absorb more toxic than filamentous LBs or cytoplasmic inclusions (27). Interestingly, the promotion of the formation of lipid-binding proteins upon proteasomal inhibition. In conclusion, Glup, a gene product adjacent to the parkin gene, attenuated cell death by Pael-R overexpression, facilitating the formation of cytoplasmic inclusions that appears to be cell-protective, at least in the short term. Thus, Glup could serve as a cell death inhibitor for degenerating neurons in PD or in related neurodegenerative disorders.

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REFERENCES
1. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstone, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., De Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
2. Kruger, R., Kuhn, W., Muller, T., Wistalla, D., Graeber, M., Koel, S., Przuntek, H., Eppen, J. T., Schols, L., and Russ, O. (1998) Nat. Genet. 15, 106–108
3. Trojanowski, J. Q., Goedert, M., Iwatsubo, T., and Lee, V. M. (1998) Cell Death Differ. 5, 832–837
4. Kitada, T., Asakawa, S., Hattori, N., Matsumura, H., Yamamura, Y., Minoshima, S., Yokohi, M., Mizuno, Y., and Shimizu, N. (1998) Nature 392, 665–668
5. Mizuno, Y., Hattori, N., and Matsumura, H. (1998) J. Neurochem. 71, 893–902
6. Shimura, H., Hattori, N., Kudo, S., Yoshikawa, M., Kitada, T., Matsumura, H., Asakawa, S., Minoshima, S., Yamamura, Y., Shimizu, N., and Mizuno, Y. (1999) Ann. Neurol. 45, 668–672
7. Imai, Y., Soda, M., and Takahashi, R. (2000) J. Biol. Chem. 275, 35661–35664
8. Shimura, H., Hattori, N., Kudo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwas, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) Nat. Genet. 25, 302–305
9. Zhang, Y., Gao, J., Chang, K. K., Huang, H., Dawson, L. V., and Dawson, T. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13354–13359
10. Auluck, P. K., Chan, H. Y. E., Trojanowski, J. Q., Lee, V. M.-Y., and Bonini, N. M. (2002) Science 295, 856–860
11. Asakawa, S., Truneman, K., Takayanagi, A., Sasaki, T., Shimizu, A., Shintani, A., Kawasumi, K., Mungall, A. J., Beck, S., Minoshima, S., and Shimizu, N. (2001) Biochem. Biophys. Res. Commun. 286, 863–868
12. West, A. B., Lockhart, P. J., O’Farrell, C., and Farrer, M. J. (2003) J. Mol. Biol. 332, 11–19
13. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001) Cell 105, 891–902
14. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) Mol. Cell 8, 613–621
15. Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K. I., and Takahashi, R. (2002) Mol. Cell 10, 55–67
16. Yaffe, M. B., Farr, G. W., Miklos, D., Harwich, A. L., Sternlicht, M. L., and Sternlicht, H. (1992) Nature 358, 245–248
17. Sternlicht, H., Farr, G. W., Sternlicht, M. L., Driscoll, J. K., Willison, K., and Yaffe, M. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9422–9426
18. Chen, X., Sullivan, D. S., and Hufnaker, T. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9111–9115
19. Gao, Y., Thomas, J. O., Chow, R. L., Lee, G. H., and Cowan, N. J. (1992) Science 256, 1043–1050
20. McNaught, K. S., Shashidharan, P., Perl, D. P., Jenner, P., and Olanow, C. W. (2002) Eur. J. Neurosci. 16, 2176–2184
21. Schlossmacher, M. G., Frosch, M. P., Gai, W. P., Medina, M., Sharn, N., Forno, L., Ochshu, T., Shimizu, H., Sharron, R., Hattori, N., Langston, J. W., Mizuno, Y., Hymun, D. B., Selkoe, D. J., and Kosik, K. S. (2002) Am. J. Pathol. 160, 1655–1667
22. 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) Nat. Med. 7, 1144–1150
23. Junn, E., Lee, S. S., Suhr, U. T., and Mouradian, M. M. (2002) J. Biol. Chem. 277, 47870–47877
24. West, A., Farrer, M., Petrucelli, L., Coin, L., Lockhart, P., and Hardy, J. (2003) J. Neurochem. 87, 1146–1152
25. Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kirmiya, T., Koid, R., Nozaki, S., Sano, Y., Ishiguro, H., Sakoe, K., Oshima, T., Satoh, T., Ikeuchi, T., Ogawa, M., Satoh, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takayama, Y., Nishizawa, M., Goto, T., Kanazawa, I., Davison, I., Tanese, N., Takahashi, H., and Tsuji, S. (2000) Nat. Genet. 26, 305–36
26. Bence, N. F., Sampath, R. M., and Kipot, R. R. (2001) Science 292, 1552–1555
27. Goldberg, M. S., and Lansbury, P. T. (2001) Nat. Cell Biol. 2, E115–E119
28. Kim, S., Nellen, E. A., Kitagawa, K., Bindokas, V. P., and Morimoto, R. I. (2002) Nat. Cell Biol. 4, 826–831
29. Sharon, R., Goldberg, M. S., Bar-Josef, I., Betensky, R. A., Shen, J., and Selkoe, D. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 99, 9110–9115
30. Nagatsu, T., Kita, K., Park, E. S., Kubota, M., Mikoshida, K., and Miyawaki, A. (2002) Nat. Biotechnol. 20, 87–90
31. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7877–7882