Parkin, the most commonly mutated gene in familial Parkinson’s disease, encodes an E3 ubiquitin ligase. A number of candidate substrates have been identified for parkin ubiquitin ligase action including CDCrel-1, α-glycosylated α-synuclein, Pael-R, and synphilin-1. We now show that parkin promotes the ubiquitination and degradation of an expanded polyglutamine protein. Overexpression of parkin reduces aggregation and cytotoxicity of an expanded polyglutamine ataxin-3 fragment. Using a cellular proteasome indicator system based on a destabilized form of green fluorescent protein, we demonstrate that parkin reduces proteasome impairment and caspase-12 activation induced by an expanded polyglutamine protein. Parkin forms a complex with the expanded polyglutamine protein, heat shock protein 70 (Hsp70) and the proteasome, which may be important for the elimination of the expanded polyglutamine protein. Hsp70 enhances parkin binding and ubiquitination of expanded polyglutamine protein in vitro suggesting that Hsp70 may help to recruit misfolded proteins as substrates for parkin E3 ubiquitin ligase activity. We speculate that parkin may function to relieve endoplasmic reticulum stress by preserving proteasome activity in the presence of misfolded proteins. Loss of parkin function and the resulting proteasomal impairment may contribute to the accumulation of toxic aberrant proteins in neurodegenerative diseases including Parkinson’s disease.

Parkin substrates have little sequence or functional similarities; however, Pael-R and α-synuclein have a propensity to misfold and aggregate (2, 4). This common property of known parkin substrates suggests that parkin may play a general role in the degradation of misfolded proteins, which might otherwise overwhelm the ubiquitin-proteasome system (UPS).

Parkin has been demonstrated to function in the endoplasmic reticulum-associated degradation (ERAD) of misfolded ER proteins (2, 6). Parkin is up-regulated during the unfolded protein response (6). Pael-R overexpression results in ER accumulation of the protein, causing ER stress-induced cell death; parkin overexpression ameliorates these effects (2). Proteasome function is also important for normal ERAD and proteasomal dysfunction can cause ER stress (7, 8). Whereas ERAD is an important pathway for eliminating misfolded proteins in the ER, there are many misfolded aggregation-prone proteins that are translated in the cytosol including most of the polyglutamine (poly(Q)) containing proteins. Accumulation of misfolded cytosolic and ER-translated proteins can ultimately inhibit proteasomal activity (9–11).

Whereas the cytotoxicity of expanded poly(Q) proteins may be because of a variety of mechanisms (12–16), expanded poly(Q) proteins impair proteasome function (9–11). Proteasomal dysfunction has also been demonstrated in PD brain and may play a role in the accumulation of aberrant proteins and neuronal loss that characterize several of the adult neurodegenerative diseases (17). Accumulation of aberrant proteins is a hallmark of both PD and the poly(Q) expansion diseases, which include Huntington’s disease (HD) and several spinocerebellar ataxias. Overexpression of aberrant proteins has been very useful for identifying genes and proteins capable of modifying their accumulation or toxicity. Molecular chaperones such as Hsp70 improve cell viability (18–20) and facilitate the elimination of poly(Q) proteins in cellular models and ameliorate disease phenotype in transgenic Drosophila models (18, 21–25). Hsp70 also improves the phenotype in a Drosophila PD model in which human α-synuclein is overexpressed (26).

Parkin appears to interact with Hsp70 along with the ubiquitinating factor CHIP (27). The N terminus of parkin contains a domain homologous to ubiquitin called the ubiquitin-like (Ubl) domain. A similar domain in the human homologue of the yeast DNA repair factor (hRad23) has been shown to interact with expanded poly(Q) proteins (28) and bind the proteasome (29, 30). Other Ubl domain containing proteins such as Dsk2 (31, 32) and Upb6 (33) also bind the proteasome. These findings suggest that parkin may also bind expanded poly(Q) proteins and proteasomes via its Ubl domain.

In the current investigations we have chosen to use an expanded poly(Q) protein as a model for cellular pathology mediated by misfolded proteins more generally. The relationship...
between genetically determined neurodegeneration and abnor-
mal misfolding of a disease causing protein is well established
in poly(Q) diseases (34). Poly(Q)-mediated neurodegeneration
is likely to serve as a model for a number of neurodegenerative
disorders in which genetic mutations of the disease-related
proteins causes misfolding and aggregation such as in
α-synuclein, SOD1, and Tau mutations (35–37). Investigations
of the role of parkin in facilitating the degradation of misfolded
proteins may also be relevant to sporadic PD and several of the
neurodegenerative conditions (38, 39).

In this study, we address whether parkin promotes the
ubiquitination and degradation of expanded poly(Q) proteins,
thus reducing impairment of the UPS. We also examine the
interaction of parkin with expanded poly(Q) proteins, Hsp70, and
the proteasome. Our goal was to further understand the
role of Hsp70 binding in parkin function and the role of
parkin in preventing cell death induced by misfolded pro-
teins. Proteasomal dysfunction can result in ER stress-
related cell death because retrotranslocation of misfolded
proteins from the ER requires ongoing ubiquitination and
proteasome function in the cytosol (8, 40). For this reason, we
assess the effects of parkin overexpression not only on UPS
function but also on the activation of pathways involved in
ER stress-induced cell death.

MATERIALS AND METHODS
Plasmid Construction and Protein Expression
Total RNA was extracted from cultures of human embryonic kidney-
derived 293 (HEK293) cells using TRIzol reagent (Invitrogen). Oli-
gotid(T)-primed first strand cDNAs were generated using Thermo-
Script™ reverse transcriptase-PCR system (Invitrogen). Polymerase
chain reaction (PCR) amplification of parkin cDNA was performed using
parkin-specific primers. Parkin mutants were generated by PCR
with wild-type parkin cDNA as template and cloned into the mamma-
lian expression vectors pcDNA3.1 (+) (Invitrogen) and CMV-FLAG 7.1
(Sigma). Wild type and mutant parkin were cloned into pGex-2T
(Amersham Biosciences) and pRSETA vector (Invitrogen). GST-E6AP,
GST-parkin, and mutants were produced in the BL21 strain of Esche-
richia coli and purified with glutathione-Sepharose 4B (Amersham Biosciences).
His6-parkin was expressed in BL21(DE3) bacteria and
purified with TALON resin (Clontech). Poly(Q) proteins (Gln26
and Gln79) fused to green fluorescent protein (GFP) were generated using a
fragment of ataxin-3 N-terminal with 72 glutamine repeats as a model for Huntington’s
disease in poly(Q) diseases (34). Poly(Q)-mediated neurodegeneration
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disorders in which genetic mutations of the disease-related
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and Gln79) fused to green fluorescent protein (GFP) were generated using a
fragment of ataxin-3 N-terminal with 72 glutamine repeats, respectively.
The ataxin-3 fragments were generated by PCR and subcloned into
EGFP-C1 vector (Clontech) leaving 44 amino acids of ataxin-3 N-ter-
minal to the poly(Q) tract and 26 amino acids at the C terminus.
GST-E6AP (originally from A. Weissman) and GST-parkin (original-
ly from D. Bohman (41)) cDNAs were generous gifts from Cecile Pickart
(Johns Hopkins). GFPU plasmid was kindly provided by Ron Kopito
(Johns Hopkins). IgG, mouse was used as control IgG for nonspecific co-immunoprecipitation.

Immunofluorescence
The YAC72 (42) transgenic mice, which express human Huntingtin
with 72 glutamine repeats as a model for Huntington’s disease, were
used in this study. Brains from 12–15-month-old YAC72 transgenic
mice were removed after perfusion with 4% paraformaldehyde and
fixed overnight in 2% paraformaldehyde and 30% sucrose in phosphate-
buffered saline (PBS). Brains were sectioned in series of 10 20-μm
coronal sections on a cryostat and collected in PBS (pH 7.5). Immunofluorescence
detection in cultured cells was performed as described in
Ref. 43. The following antibodies were used: anti-FLAG M2 (Sigma),
anti-HA (Clontech), anti-parkin (Cell Signaling Technologies), anti-
Huntingtin (Chemicon), and anti-ubiquitin (generous gift from Cecile
Pickart). Cy5-conjugated donkey anti-rabbit and fluorescein isothiocya-
nate or Cy3-conjugated donkey anti-mouse were used as secondary
antibodies (Chemicon). Controls included omission of primary antibody
or primary antibody alone. As additional controls of parkin immunofluo-
rescence, poly(Q)-containing proteins from the ER were sectioned on a cryostat in 10-
μm sections and collected in PBS. Immunofluorescence was performed using anti-Huntingtin (Chemicon)
and anti-parkin antibodies HP2A recognizing amino acids 342–353 of
parkin (a generous gift of Michael Schlossmacher). The HP2A antibody
has been successfully used on human brain autopsy samples to demon-
strate localization of parkin to Lewy bodies (44). Fluorescein isothio-
cyanate-conjugated donkey anti-mouse and Cy5-conjugated donkey
anti-rabbit were used as secondary antibodies (both from Chemicon).

Immunoprecipitation and Immunoblot Analysis
Transfected cells were harvested, washed in PBS, and lysed in lysis
buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl
fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5–1.0% Triton
X-100). Lysates were centrifuged at 15,000 × g for 10 min, and the
supernatant was precleared before immunoprecipitation. Samples (300 μg)
were incubated with 40 μl of anti-FLAG M2 affinity gel (Sigma) at
4 °C for 2 h with constant mixing. The immobilized immunocomplexes
were collected by centrifugation, washed 3× with lysis buffer, and
boiled in SDS sample buffer for SDS-PAGE. For brain homogenates,
mouse brains were removed, homogenized in lysis buffer containing
2 mM ATP and 1% Nonidet P-40 with a Dounce homogenizer. Brain
homogenates were centrifuged at 15,000 × g for 10 min at 4 °C and used
for immunoprecipitation (1 mg/ml) as described above using anti-
Huntingtin (Chemicon) or anti-parkin antibodies (Cell Signaling Technolo-
gies or HP2A kindly provided by Michael Schlossmacher). Rabbit anti-
mouse was used as control IgG for non-specific co-immunoprecipitation.

The antigen complex was eluted and processed for SDS-PAGE. For
immunoblots, cells were lysed in lysis buffer and briefly sonicated.
Equal amounts (50 μg) of cell lysates were separated by SDS-PAGE,
transferred to membranes, probed with the appropriate antibody,
and visualized using chemiluminescence. GFP-Gln26, GFP-Gln79, and GFP
were detected with rabbit polyclonal anti-FLAG (Clontech). FLAG-
tagged wild-type and mutant parkin were detected using anti-FLAG
M2 (Sigma). HA-tagged poly(Q) proteins were detected with anti-HA
(Clontech). Caspase-12 was detected by rabbit polyclonal anti-
caspase-12 (Cell Signaling Technologies). The Rpt6/S8 and HC3 sub-
units of the proteasome were detected with rabbit polyclonal antibodies
against p45 and HC3, respectively (Affiniti).

Confocal Microscopy and Quantification of Aggregates
and Cell Viability
All images were acquired on a Zeiss LSM 510 confocal microscope.
Images were minimally processed for presentation. In all experiments
in which aggregates and cell viability were quantified, the same
observer, blinded to the transfection, scored the cells. For GFP-Gln26
aggregates, cells with large visible inclusions were counted (see Fig.
4A). Cell viability was assessed by propidium iodide exclusion or
fluorescence microscope (Zeiss Axiovert). Only cells expressing GFP-
poly(Q) proteins were scored for propidium iodide exclusion. Transfec-
tion efficiency is about 50%. For each experiment, 200-500 cells were
counted for each treatment.
Parkin Preserves Proteasome Function

In Vitro Ubiquitination of Expanded Poly(Q) Proteins—35S-Labeled

Parkin interacts with expanded poly(Q) protein in brains of YAC72 transgenic mice. A, colocalization of parkin with poly(Q)-expanded Huntingtin in brain of HD transgenic mice. Coronal sections of forebrains from YAC72 HD transgenic mice were prepared for immunofluorescence as described under "Materials and Methods." Immunofluorescence of Huntingtin is shown in green. Parkin (red in upper panel) colocalizes with Huntingtin inclusions, which also contain ubiquitin (lower panel). Yellow in the Merge images indicates colocalization. Scale bar, 20 μm. B, co-immunoprecipitation of Huntingtin with parkin from transgenic mouse brain. Left, immunoprecipitates of parkin antibody or a control IgG were analyzed for Huntingtin (upper panel) or parkin (lower panel) by Western blot. "Input" shows 50% of the starting extract. F1 and F3 represent brain homogenates from 15- and 9-month-old females, respectively. Right, parkin was immunoprecipitated from brain extracts prepared from HD transgenic (F1) or non-transgenic control (NC) animals. Immunoprecipitates were analyzed for Huntingtin (upper panel) or parkin (lower panel). WB, Western blot.

Ubiquitination Assays

Auto-ubiquitination—FLAG-tagged wild-type and mutant parkin expressed in HEK293 cells were immunoprecipitated using anti-FLAG M2 antibody (300 μg, described above). The ubiquitination reaction contained the immunocomplexes, mammalian ubiquitin-activating enzyme E1 (70 nM), the E2 ubiquitin-conjugating enzyme UbcH7 (100 nM), and 35S-labeled ubiquitin (5 μM) in a reaction buffer of 50 mM Tris (pH 7.6), 5 mM MgCl2, 2 mM ATP with an ATP regenerating system. The reaction (50 μl) was incubated at 37 °C with gentle agitation for the indicated period of time and quenched with 2× SDS sample buffer (2).

In Vitro Ubiquitination of Expanded Poly(Q) Proteins—35S-Labeled ataxin-3 Gln79 was translated in vitro using either a rabbit reticulocyte lysate or S30 T7 bacteriophage lysate system (Promega). Ubiquitination reactions contained 1 μl of translation mixture, mammalian E1 (100 nM), UbcH7 (300 nM), bovine ubiquitin (5 μM, Sigma), E2 (GST-E6AP, GST-parkin or mutants, 500 nM) in 50 μl of reaction buffer (above). Hsp70 (1 μg, Sigma) was added where indicated. The reaction was incubated for 2 h at 37 °C and quenched with 2× SDS sample buffer. The reaction mixtures were separated by SDS-PAGE and processed for visualization on a Storm PhosphorImager (Amersham Biosciences).

Pulse-Chase Experiments

HEK293 cells were transfected with the indicated expression plasmids and cultured for 30 h. Cells were washed and starved in Met/Cys-free medium for 1 h before labeling with 50 μCi/ml [35S]Met and [35S]-Cys for 1 h (Promix, Amersham Biosciences). After labeling, cells were washed three times and chased in normal medium supplemented with unlabeled Met and Cys. Where indicated, 50 μM MG132 was added to the labeling and chase medium to inhibit proteasome activity. At the indicated times, cells were washed twice with PBS, lysed in RIPA buffer, and briefly sonicated before immunoprecipitation with anti-GFP antibody. Immunocomplexes were washed, boiled in SDS sample buffer, and separated by SDS-PAGE. Radiolabeled proteins were visualized by exposure to phosphorimage screens and analyzed with a Storm PhosphorImager (Amersham Biosciences).

Assay for the Ubiquitin-Proteasome System in GFPu Cells

HEK293 cells stably expressing GFPu were transiently transfected with HA-Gln79 and FLAG-tagged wild-type or mutant parkin. After 72 h, cells were imaged for GFPu fluorescence and HA-Gln79 and FLAG-parkin expression (immunofluorescence). For cells transfected with HA-Gln79, GFPu fluorescence of individual cells expressing HA-Gln79 was measured. To evaluate the effect of overexpressing parkin on GFPu fluorescence, GFPu fluorescence of cells expressing FLAG-parkin were measured.

In Vitro Binding Assays

An aliquot containing 10 μg of GST, GST-parkin, or GST-parkin mutants immobilized on glutathione-Sepharose 4B was incubated with 0.1 μg purified bovine 26 S proteasomes (gift of Y. Lam, Johns Hopkins (45)) in 20 μl of binding buffer (50 mM Tris, pH 7.6, 1 mM dithiothreitol, 1 mM ATP) for 2 h at 4 °C with constant mixing. The immobilized proteins were collected by centrifugation, washed 3× with binding buffer, and boiled in SDS sample buffer for SDS-PAGE (2). HA-Gln79 was translated in vitro using S30 T7 bacteria lysate. An aliquot of the translation mixture was incubated with His6-parkin (1 μg) in 25 μl of buffer containing 50 mM Tris (pH 7.6), 1 mg/ml ovalbumin for 2 h at room temperature with constant mixing. HA-Gln79 was immunoprecipitated with anti-HA antibody and His6-parkin immunoprecipitated with anti-His6 antibody (Clontech).

Statistical Analysis

Non-parametric statistics were used in this study to avoid assumptions about the underlying distributions of the data. The Kruskal-Wallis test was used for multiple comparisons and p < 0.05 considered statistically significant. For two-sample comparison, treatment was compared with control (transfection with empty vector) using two-tailed Wilcoxon signed-rank test. Data were analyzed with S-Plus and presented as mean ± S.D.

RESULTS

Parkin Interacts with Expanded Poly(Q) Proteins in Vivo and in Cells—Immunofluorescence revealed that parkin was localized in the Huntingtin (htt)-containing aggregates in brains of YAC72 transgenic mice (42), which express human htt with 72 glutamines under the control of the htt promoter (Fig. 1A). The aggregates also contained ubiquitin (Fig. 1A). We next determined if parkin colocalized to htt-containing inclusion bodies in human brain tissue from HD patients (Fig. 2). Immunofluorescence showed that parkin was localized to both cytoplasmic and nuclear inclusions of htt found in the caudate and frontal cortex of HD brains. Pre-adsorption of the parkin antibody eliminated parkin immunofluorescence suggesting that parkin colocalized...
Parkin preserves proteasome function

with inclusions containing poly(Q)-expanded htt in HD brain and in transgenic mouse models of HD.

We next examined if parkin interacts with the poly(Q)-expanded htt in the soluble fraction by co-immunoprecipitation (Fig. 1B). Parkin immunoprecipitates from YAC72 brain homogenates contain htt, showing that parkin interacts with htt in vivo. To determine whether the state of htt aggregation affected its interaction with parkin, immunoprecipitation of parkin was performed from brain homogenates of 9-month-old (before aggregation occurs) and 15-month-old YAC72 transgenic mice, which contain htt inclusions (42). The age of the animal had no effect on the co-immunoprecipitation of parkin with htt, demonstrating that the state of aggregation of htt does not affect the interaction of parkin and htt (Fig. 1B, left). Htt was absent from parkin immunoprecipitates from non-transgenic control brain (Fig. 1B, right). Because the YAC72 mice express human htt at only one-third to one-half the level of endogenous htt (42), the interaction of parkin with poly(Q)-expanded htt in these animals is unlikely to be an artifact of overexpression of the human htt. The htt antibody used in this experiment recognizes both mouse and human htt. These results suggest that parkin interacts preferentially with poly(Q)-expanded htt.

To determine whether parkin interacts with poly(Q)-expanded proteins in cells, we used a fragment of ataxin-3 containing Gln79 fused to GFP (GFP-Gln79). Human embryonic kidney-derived 293 (HEK293) and mouse neuroblastoma N18 cells overexpressing GFP-Gln79 develop fluorescent aggregates located in the cytoplasmic, perinuclear, or intranuclear regions (see Figs. 3 and 5). Immunofluorescence analysis in N18 cells showed that endogenous parkin colocalized with GFP-Gln79 in these aggregates (Fig. 3A), even though it is normally expressed in the cytoplasm and excluded from the nucleus (Fig. 3B). We next examined if parkin interacts GFP-Gln79 in the soluble fraction by co-immunoprecipitation. HEK293 cells were transfected with FLAG-tagged parkin and GFP-Gln79. Immunoprecipitates of FLAG-tagged parkin contain GFP-Gln79 showing that parkin interacts with GFP-Gln79 in cells (Fig. 3C, left). We next determined if parkin interacts preferentially with expanded poly(Q) proteins in our model. HEK293 cells were transfected with FLAG-tagged parkin together with GFP, GFP-Gln26, or GFP-Gln79. Immunoprecipitation of FLAG-tagged parkin from cell lysates preferentially co-precipitated GFP-Gln79 in comparison with GFP-Gln26, showing that parkin preferentially interacts with Gln79 in these cells (Fig. 3C, right, compare lanes 1 and 2). Immunoprecipitation of FLAG-parkin did not co-precipitate GFP indicating that the interaction was specific for the poly(Q) region.

Parkin promotes the ubiquitination and degradation of an expanded poly(Q) protein—To establish a functional role for FLAG-tagged parkin, we immunoprecipitated FLAG-tagged parkin from HEK293 cell lysates and examined its E3 ubiquitin ligase activity in vitro (Fig. 4A). Immunoprecipitated FLAG-parkin showed autoubiquitination in vitro in the presence of an E1 and UbcH7 (Fig. 4A). The disease-associated parkin truncation mutant, Q311X, on the other hand, did not show any E3 activity in this assay (lane 4), consistent with previous reports (1, 3, 6). Interestingly, a single mutation of a critical cysteine residue of the distal RING (C418R) completely abolished parkin E3 activity (Fig. 4A, lane 5), suggesting the two RING fingers may function as a single motif (46).

Because parkin exhibits ubiquitin ligase activity and interacts with GFP-Gln79, it is natural to ask if parkin can ubiquitinate the expanded poly(Q) protein. To address this question, we prepared [35S]-labeled ataxin-3 Gln79 by in vitro translation and tested if bacterially produced GST-parkin promoted its ubiquitination (Fig. 4B). As shown in Fig. 4B, wild-type parkin but not the Q311X or C418R parkin mutants, promoted the ubiquitination of Gln79. Although monoubiquitinated Gln79 was the predominant product, more polyubiquitinated forms of Gln79 were also produced (for example, lane 8). Previous genetic studies suggested that E6AP promotes the ubiquitination of a different poly(Q)-expanded protein, ataxin-1 (47). As a control, we determined if E6AP could promote the ubiquitination of Gln79 in vitro. Contrary to our expectation, E6AP did not promote ataxin-3 Gln79 ubiquitination in our in vitro assay (Fig. 4B, right panel).

The best known function of protein ubiquitination is to target proteins for degradation by the 26 S proteasome. We therefore assessed if parkin facilitated the degradation of GFP-Gln79 in cultured cells. Pulse-chase experiments showed that co-expression of parkin accelerated the degradation of GFP-Gln79 (Fig. 4C, • versus ▲) and the effect could be blocked with a proteasome inhibitor MG132 (• versus ◊). Co-expression of E6AP or the parkin mutant Q311X had no detectable effect on the rate of turnover of GFP-Gln79 (Fig. 4C), in agreement with the failure of these proteins to promote GFP-Gln79 ubiquitination in vitro (Fig. 4B). These data show that parkin promotes the degradation of GFP-poly(Q) proteins via the ubiquitin-proteasome pathway.

Parkin suppresses aggregation and toxicity of GFP-Gln79—Based on the results presented above, we considered it possible that parkin could protect cells from deleterious effects of overexpressing expanded poly(Q) proteins. N18 cells transiently transfected with GFP-Gln79 developed fluorescent aggregates (Fig. 5A), as previously seen for this and other expanded poly(Q) proteins (9, 11, 13, 15, 18–20, 48–50). Using confocal microscopy, we assigned transfected N18 cells to one of three groups: cells with 1) large fluorescent inclusions; 2) small mul-
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Fig. 3. Interaction of parkin with GFP-poly(Q) proteins in cultured cells. A, colocalization of parkin with GFP-Gln79 in N18 cells. N18 rat neuroblastoma cells were transfected with GFP-Gln79. GFP-Gln79 was visualized by GFP fluorescence (green) and endogenous parkin by anti-parkin immunofluorescence (red). A fraction of the cells developed cytoplasmic and nuclear aggregates, which contained both GFP-Gln79 and parkin. Scale bar, 10 μm. B, distribution of endogenous parkin in untransfected N18 neuroblastoma cells by immunofluorescence. C, co-immunoprecipitation of GFP-Gln79 with parkin. Left, HEK293 cells were transfected with FLAG-parkin and GFP-Gln79. Cell lysates were processed for immunoprecipitation with anti-FLAG antibody. Pre-adsorption of anti-FLAG antibody with 3× FLAG peptide prevented immunoprecipitation of FLAG-parkin. Right, HEK293 cells were transfected with FLAG-parkin and GFP-Gln79 (lanes 1 and 4); GFP-Gln79 (lanes 2 and 5), or GFP (lanes 3 and 6). Cell lysates were processed for immunoprecipitation with anti-FLAG antibody and immunoblotted for GFP (upper panel) or Fll (lower panel). WB, Western blot.

Multiple inclusions; or 3) diffuse fluorescence (Fig. 5A). Co-transfection of FLAG-parkin with GFP-Gln79 significantly reduced the percentage of cells showing large inclusions (Fig. 5B), in a manner that was dependent on the amount of parkin cDNA transfected (Fig. 5B, left). The parkin-dependent reduction in aggregate size was greatly diminished in the presence of the proteasome inhibitor MG132, whereas MG132 only minimally increased aggregate size in the absence of co-expressed parkin (Fig. 5B, right). No parkin-dependent reduction in GFP-Gln79 aggregation occurred upon co-transfection of the parkin mutants Q311X and C418R (Fig. 4C); nor did co-expression of two other E3s, E6AP and XIAP, mimic the effect of wild-type parkin. These results are consistent with the failure of the parkin mutants and E6AP to promote GFP-Gln79 ubiquitination (Fig. 4B) or degradation (Fig. 4C). These results thus suggest that wild-type parkin inhibits the formation of GFP-Gln79 inclusions by promoting the ubiquitination and degradation of the poly(Q) protein. Consistent with this interpretation, immunoblot analysis showed that overexpression of wild-type parkin, but not mutant Q311X, reduced the steady-state level of GFP-Gln79 (Fig. 5D, lanes 2–4 versus 5) and this effect was blocked by MG132 (lane 6). The failure of parkin mutant Q311X to reduce GFP-Gln79 levels was not because of poor expression (Fig. 5D, lower panel).

To investigate if parkin protects against toxicity of expanded poly(Q) proteins, we co-expressed FLAG-tagged parkin with GFP-Gln79 in N18 cells. The ratio of parkin to GFP-Gln79 plasmid DNA was varied (while keeping the total amount of DNA constant with empty vector) to investigate the dose effect of parkin overexpression. Co-expression of wild-type parkin significantly enhanced cell viability as assayed by propidium iodide exclusion (Fig. 6A). Adding MG132 greatly reduced the protective effect of parkin indicating that its effects on protein aggregation and cell survival both depend on the ubiquitin-proteasome pathway. Co-expression of parkin mutants Q311X or C418R showed no protective effect (Fig. 6B). Co-expression of two other E3s, E6AP and XIAP, offered no protection against GFP-Gln79 toxicity (Fig. 6B), indicating that protection in this model system is specific to enzymatically active parkin rather than a general property of overexpression of any ubiquitin ligase. It is reasonable to propose that this protection relies on the parkin-dependent ubiquitination and degradation of GFP-Gln79 documented in Figs. 4 and 5 above.

Parkin Preserves the Ubiquitin-Proteasome System from Impairment by an Expanded Poly(Q) Protein—Expanded poly(Q) proteins have been shown to inhibit the proteasome that leads to ER stress contributing to expanded poly(Q)-mediated cell death (9–11). To study the effect of parkin overexpression on proteasome function, we generated HEK293 cell lines stably expressing GFPu, a form of GFP that is selectively targeted to proteasomes, as shown by a 6–7-fold increase in GFPu fluorescence following treatment with 10 μM MG132 for 6 h (Fig. 7A, panel 6; see also Ref. 9). Transient transfection of HA-Gln79 in GFPu cells increased GFPu fluorescence by 7–8-fold (Fig. 7, A, second panel, and B) confirming a previous demonstration of proteasome inhibition by overexpression of a poly(Q)-expanded htt exon 1 (9). Co-expression of wild-type parkin reduced the increase in GFPu fluorescence caused by HA-Gln79 overexpres-
sion, resulting in a distribution that was closer to the control lacking HA-Gln79 expression (Fig. 7, A, third versus fourth panels; see also Fig. 7C). Co-expression of the inactive C418R-parkin mutant failed to ameliorate the increase in GFPu fluorescence caused by HA-Gln79 overexpression (Fig. 7, B, fourth panel; see also Fig. 7C). Importantly, overexpression of parkin alone did not significantly decrease GFPu fluorescence showing that parkin did not directly target GFPu for degradation (Fig. 7C). The reduction in GFPu fluorescence observed when parkin was co-expressed with HA-Gln79 therefore indicated improved UPS function in these cells. This improvement in UPS function results from parkin-facilitated degradation of expanded poly(Q) proteins.

**Parkin Reduces Caspase-12 Activation**—Proteasome function is essential for normal ERAD and relief of ER stress (7, 8). Overexpression of poly(Q) containing proteins has been shown to induce ER stress by inhibiting proteasome activity (12, 51). Blocking ER stress-mediated apoptosis via the ASK pathway prevents the neuronal death induced by poly(Q) overexpression (51), suggesting that ER stress resulting from poly(Q) inhibition of the proteasome may be important in poly(Q)-mediated cell death. To determine whether the ability of parkin to preserve proteasome function in the setting of poly(Q) overexpression translates into reduced ER stress, we monitored caspase-12 activation as a marker for ER stress. Activation of caspase-12 in murine neurons is an important effector pathway for ER stress-induced neuronal cell death (52). HEK293 cells co-transfected with procaspase-12 and GFP-Gln79 plasmids showed increased activation of caspase-12 (Fig. 8, lanes 1 and 2) and cell death (data not shown). Co-expression of wild-type parkin, but not the inactive mutant parkin-Q311X, reduced the activation of caspase-12 (Fig. 8, lanes 3 and 4). These results suggest that parkin can protect cells against ER stress-induced cell death by improving proteasome function and consequently maintaining ERAD, which reduces ER stress.

**Parkin Interacts with Hsp70 and the Proteasome**—The molecular chaperone Hsp70 has been previously shown to reduce aggregation of expanded poly(Q) proteins (18, 19, 22, 23, 53). Hsp70 is also involved in the degradation of certain misfolded proteins (21, 54–56). We have shown that parkin interacts with, and promotes the degradation of, misfolded poly(Q) proteins. We therefore tested if parkin interacts with Hsp70. We found that antibody against FLAG-parkin co-precipitated Hsp70 (Fig. 9A, top panel), confirming a previous report by Imat et al. (27) that parkin forms a complex with Hsp70 in cells. In contrast, Hsp70 did not co-precipitate with a different E3

![Image](https://example.com/image.png)
Fig. 5. Parkin reduces aggregation of GFP-poly(Q) proteins. A, GFP-Gln79 inclusion body formation in N18 cells. GFP-Gln79 was expressed by transfection (see “Materials and Methods”). Confocal images representative of the three types of inclusions (see text) are shown. B, parkin reduces GFP-Gln79 aggregation. Left, N18 cells were co-transfected with GFP-Gln79 and empty vector (control) or varying quantities (0.5, 1.0, and 2.0 μg) of FLAG-parkin. Right, cells co-transfected with empty vector and 2.0 μg of FLAG-parkin were cultured in the presence of a proteasome inhibitor MG132 (added 48 h post-transfection). These experiments were performed together but presented on two panels for clarity. C, specificity of parkin-dependent reduction in aggregate size. N18 cells were co-transfected with GFP-Gln79 and empty vector, FLAG-parkin, the indicated parkin mutants, E6AP and XIAP, and assayed for GFP-Gln79 aggregation. Data shown are means with error bars indicating S.D. (n = 9; *, p < 0.01; **, p < 0.005 compared with control transfection with empty vector). D, parkin reduces steady state level of GFP-Gln79. HEK293 cells were transfected with GFP-Gln79 and varying quantities (0, 0.5, 1.0, and 2.0 μg) of parkin or 2.0 μg of mutant Q311X. 50 μg of total cell lysate were analyzed by Western blot for GFP (top panel) or FLAG epitope (bottom). Cells were cultured in 50 μM MG132 (added 48 h post-transfection) overnight in lane 6.

(E6AP, bottom panel). To determine the region of parkin involved in this interaction, we constructed FLAG-tagged deletion mutants of parkin and examined their interaction with Hsp70 by co-immunoprecipitation from lysates of cells transfected with FLAG-parkin and GFP-Gln79 (Fig. 9B). The results revealed that an intact RING-IBR-RING region was essential for Hsp70 interaction, consistent with a previous report of Hsp70 binding to parkin-Pael-R complex (27). Because Hsp70 has been previously shown to interact with misfolded proteins including expanded poly(Q) proteins (11, 18–21, 23, 53, 57), co-immunoprecipitation of Hsp70 and parkin could reflect either a direct interaction between parkin and the expanded repeat protein, or an indirect interaction with a complex containing Hsp70 together with the expanded repeat protein.

The interaction of parkin with Hsp70 may be functionally important in at least two ways. Hsp70 may enhance the binding and ubiquitination of the substrate by parkin similar to Refs. 58 and 59. Alternatively, Hsp70 may inhibit the E3 activity of parkin to promote refolding of the substrate suggested in Ref. 27. To better understand the role of parkin interaction with Hsp70, we examined the interaction of parkin and HAtagged poly(Q) protein in vitro. HA-Gln79 was in vitro translated, in the presence or absence of Hsp70, using a bacterial system, and incubated with bacterially expressed His6-parkin. Hsp70 enhanced the binding of HA-Gln79 to His6-parkin (Fig. 9C, right versus left, top panels). In a parallel experiment, Hsp70 similarly enhanced the co-immunoprecipitation of His6-parkin with HA-Gln79 (Fig. 9C, bottom panels). We next examined the effect of Hsp70 on the ability of parkin to ubiquitinate in vitro translated poly(Q)-expanded proteins. [35S]-Labeled ataxin-3 Gln79 was translated in vitro, in the absence of Hsp70, using a bacterial system and then used as a substrate in a ubiquitination assay with or without added Hsp70 (Fig. 9D). The addition of Hsp70 enhanced parkin-dependent ubiquitination of Gln79, possibly by enhancing the binding of parkin to Gln79 (cf. panel C). We did not observe this requirement in Hsp70 for in vitro ubiquitination of Gln79 translated using a reticulocyte lysate system (Fig. 4). This observation may reflect the differences between bacteria lysate and reticulocyte lysate systems; reticulocyte lysate already contains substantial amounts of many molecular chaperones (estimated at 2 μM functional Hsp70 and Hsp90 (60)). Imai et al. (27) have also observed that larger amounts of Hsp70 can inhibit parkin-dependent ubiquitination of the substrate Pael-R in vitro. Taken together, our results suggest that parkin preferentially binds and ubiquitinates misfolded poly(Q) proteins via its interaction with Hsp70.

The efficient degradation of misfolded proteins requires the efficient presentation of these substrates to the proteasome. Substrate recognition is generally dependent on polyubiquitination, but additional factors may be necessary for efficient recognition or processing in some cases. The Ubl domains of certain other Ubl proteins have been shown to bind the proteasome, an event that may recruit additional factors to the degradation machinery (29, 61–63). We therefore investigated the possibility that parkin interacts with the proteasome. We observed that Rpt6, a proteasome ATPase subunit, co-precipitates with parkin from lysates of cells co-transfected with FLAG-parkin and GFP-Gln79 (Fig. 10A). Because proteasome subunits have been found in inclusions containing expanded
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Parkin appears to be capable of protecting the proteasome from inhibition by expanded poly(Q) proteins. Our results therefore extend the observations of Imai et al. (2, 6) and suggest that parkin targets the degradation of several misfolded substrates in multiple cellular compartments.

This study also demonstrates that parkin is an E3 ubiquitin ligase for poly(Q) expanded ataxin-1. The interaction of parkin with expanded poly(Q) proteins is not simply a result of overexpression, as evidenced by the colocalization and co-immunoprecipitation of parkin with poly(Q) expanded htt in the brains of YAC72 HD transgenic mice and colocalization with htt inclusions in HD brains. These findings suggest a role for parkin function in neurodegenerative diseases such as HD. Previous genetic studies have implicated E6AP, a HECT domain E3 ubiquitin ligase, in the ubiquitination of poly(Q) expanded ataxin-1. Mutation of E6AP results in a reduction in the formation of visible inclusions but enhanced cytotoxicity in transgenic mice overexpressing expanded poly(Q) ataxin-1 (47). Although E6AP did not show E3 activity for poly(Q)-expanded ataxin-3 protein in our in vitro assay, it is possible that E6AP might specifically ubiquitinate ataxin-1 but not ataxin-3. These proteins are substantially different with respect to protein size, sequence, protein interacting partners, and subcellular localization. It is also conceivable that another factor is required for effective ubiquitination of ataxin-3 by E6AP. Alternatively, E6AP may ubiquitinate a poly(Q) interacting protein important for inclusion body formation without directly ubiquitinating the poly(Q) protein.

Several other candidate substrates for parkin have been previously identified (2–5). These candidate substrates have little sequence homology. However, the substrates Pael-R and αSp22 share with the expanded poly(Q) proteins a common propensity to misfold and aggregate (2, 4). Our results indicate that parkin preferentially ubiquititates expanded poly(Q) containing proteins. It is not known how parkin recognizes its apparently diverse protein substrates. We show that Hsp70 associates with parkin and expanded poly(Q) proteins in a complex similar to the reported complex of Hsp70, parkin, and Pael-R (27). Hsp70 binding appears to be mediated primarily by the RING-IBR-RING domain. Hsp70 enhances the binding and ubiquitination of expanded poly(Q) proteins by parkin in vitro where the expanded poly(Q) proteins are translated in a bacteria lysate system. Hsp70 is important in the recognition and unfolding of misfolded proteins and degradation of certain misfolded proteins (21, 54, 59). Hsp70 has also been shown to preferentially bind htt with expanded poly(Q) repeats compared with normal repeats (19) and promote the degradation of poly(Q)-expanded androgen receptor (21). Our results suggest that parkin may preferentially recognize misfolded poly(Q) proteins via interaction with Hsp70, which may partially account for its apparent broad substrate specificity. This result differs from that of Imai et al. (27) where adding larger amounts of Hsp70 (3–4 μg) inhibited parkin-dependent ubiquitination of Pael-R in vitro. In their assay, Pael-R was translated in rabbit reticulocyte lysate, which contains a substantial amount of Hsp70 and many other components of the UPS. The different effects of Hsp70 on parkin E3 activity may reflect differences between an ER versus cytosolic protein substrate or differences between bacteria and rabbit reticulocyte lysates or the quantity of Hsp70 added. In our model, parkin is able to recognize misfolded poly(Q) proteins by its interaction with Hsp70. This suggests that parkin may recognize a wide variety of misfolded proteins by forming a complex with Hsp70 and thus parkin may function as an E3 for misfolded proteins more broadly.

The efficient degradation of misfolded proteins may require effective presentation of these substrates to the proteasome.

**Fig. 6.** Parkin reduces toxicity of GFP-poly(Q) proteins. A, wild-type parkin reduced GFP-Gln79 toxicity in a concentration- and proteasome-dependent manner. N18 cells were co-transfected with GFP-Gln79 and empty vector (control) or increasing quantities (0.5, 1.0, and 2.0 μg) of FLAG-parkin. Where indicated, 50 μM MG132 was added overnight. These experiments were performed together but presented on two panels for clarity. B, specificity of parkin amelioration of GFP-Gln79 toxicity. N18 cells were transfected with GFP-Gln79 and 2.0 μg of parkin, and the indicated parkin mutants, E6AP or XIAP. Cells were assayed for propidium iodide exclusion 60 h post-transfection. Data shown are means with error bars indicating S.D. (n = 9; *, p < 0.01; **, p < 0.005 compared with control transfection with empty vector).

poly(Q) proteins, this interaction could be indirect (10, 18, 64). To determine whether parkin interacts directly with the proteasome, we examined binding of GST-parkin to purified 26 S proteasomes in vitro. We found that GST-parkin indeed bound directly to 26 S proteasomes as indicated by pull-down of Rpt6 (Fig. 10B, left panel). Similar assays with parkin variants identified the Ubl domain as being critical for this interaction (Fig. 10C, right panel).

**DISCUSSION**

Parkin has previously been demonstrated to function in ERAD for degrading unfolded proteins from the ER (2, 6). However, many misfolded proteins are translated in the cytosol including α-synuclein and several expanded poly(Q) proteins. In this study, we show that parkin also promotes the ubiquitination and degradation of a misfolded protein translated in the cytosol. The model misfolded protein used in this study, a poly(Q) expanded ataxin-3 fragment, misfolds in the cytosol and inhibits the proteasome. Parkin preferentially binds and facilitates the degradation of the misfolded poly(Q) protein thereby reducing the impairment of proteasome function. Ongoing proteasome activity is required for the retrotranslocation of misfolded ER proteins to the cytosol for degradation. When proteasome activity is inhibited, retrotranslocation of misfolded ER proteins fails and the ERAD mechanism for the reduction of ER stress is blocked. ER stress can then lead to the activation of apoptosis pathways including the activation of caspase-12 and ASK. Thus ER stress-mediated cell death can arise from either overexpression of a misfolded ER protein such as Pael-R or by proteasome inhibition by cytosolic translated misfolded proteins such as the poly(Q) containing proteins.

Parkin appears to be capable of protecting the proteasome from inhibition by expanded poly(Q) proteins. Our results therefore extend the observations of Imai et al. (2, 6) and suggest that parkin targets the degradation of several misfolded substrates in multiple cellular compartments.

This study also demonstrates that parkin is an E3 ubiquitin ligase for poly(Q) expanded ataxin-3. The interaction of parkin with expanded poly(Q) proteins is not simply a result of overexpression, as evidenced by the colocalization and co-immunoprecipitation of parkin with poly(Q) expanded htt in the brains of YAC72 HD transgenic mice and colocalization with htt inclusions in HD brains. These findings suggest a role for parkin function in neurodegenerative diseases such as HD. Previous genetic studies have implicated E6AP, a HECT domain E3 ubiquitin ligase, in the ubiquitination of poly(Q) expanded ataxin-1. Mutation of E6AP results in a reduction in the formation of visible inclusions but enhanced cytotoxicity in transgenic mice overexpressing expanded poly(Q) ataxin-1 (47). Although E6AP did not show E3 activity for poly(Q)-expanded ataxin-3 protein in our in vitro assay, it is possible that E6AP might specifically ubiquitinate ataxin-1 but not ataxin-3. These proteins are substantially different with respect to protein size, sequence, protein interacting partners, and subcellular localization. It is also conceivable that another factor is required for effective ubiquitination of ataxin-3 by E6AP. Alternatively, E6AP may ubiquitinate a poly(Q) interacting protein important for inclusion body formation without directly ubiquitinating the poly(Q) protein.

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The efficient degradation of misfolded proteins may require effective presentation of these substrates to the proteasome.
Substrate recognition is generally dependent on polyubiquitination, but additional factors may be necessary for efficient recognition or processing in some cases. It has also been suggested that some E3 ubiquitin ligases directly bind the proteasome, which may help to present the substrate to the proteasome (65) or recruit additional factors to the degradation machinery (62, 63). We found that parkin binds the proteasome and the Ubl domain is critical for this interaction. Recent NMR studies show that the Ubl domain of parkin interacts with the Rpn10 subunit of the proteasome in vitro and the interaction region includes Arg42 of the Ubl domain of parkin (66). A single point mutation R42P in the Ubl domain has been linked to early-onset Parkinsonism (67), suggesting that proteasome binding may be important for parkin function. The recent work of Imai et al. (27) demonstrates that parkin forms a complex that contains not only the substrate Pael-R but also Hsp70 and the ubiquitinating factor CHIP, which appears to enhance the E3 activity of parkin (27). CHIP-related polyubiquitination of substrates appears to be important for degradation of ER misfolded proteins (58, 59, 68, 69). Hsp70 has been shown to be involved in the degradation of certain misfolded proteins (21, 54, 56, 70). We speculate that parkin may interact with Hsp70 and the proteasome to facilitate direct presentation of the ubiquitinated substrate in an unfolded state more suitable for entry into the proteasomal pore for successful degradation.

Parkin improves proteasome function in the presence of misfolded cytosolic poly(Q) proteins, which can otherwise impair the UPS and activate cell death (9, 12, 51). Proteasome dysfunction occurs in the presence of misfolded ER proteins such as mutant cystic fibrosis transmembrane conductance regulator or misfolded cytosolic proteins such as expanded poly(Q) proteins and α-synuclein (9, 10, 12, 71). Whereas protein aggregation, proteasome dysfunction, and cell death appear to be closely related in cell culture models, the relationship between inclusion body formation and cell death in vivo is less clear. In transgenic mice that overexpress poly(Q)-expanded ataxin-1, mutation in the E3 ubiquitin ligase E6AP results in enhanced cytotoxicity despite a reduction in the formation of visible inclusions (47). Nevertheless, there is a growing amount of recent evidence that proteasome dysfunction and accumulation of misfolded proteins may play a role in the pathogenesis of several neurodegenerative diseases. Proteasomal subunits are found in poly(Q) protein aggregates (10, 11, 64) as well as in Lewy bodies, the hallmark intracellular inclusion of PD (72).

Fig. 7. Parkin improves proteasome function in the presence of expanded poly(Q) protein. A, HEK293 cells stably expressing GFPu were transfected with the indicated plasmids and GFPu fluorescence was quantitated on a fluorescence microscope (“Materials and Methods”). B, co-expression of HA-Gln79 and GFPu stabilizes GFPu. HEK293 cell lines stably expressing GFPu were transfected with HA-Gln79 and monitored for GFPu fluorescence and expression of HA-Gln79 (HA immunofluorescence). Arrows indicate HA-Gln79 expressing cells. C, smoothed density estimates of GFPu fluorescence for selected histograms in panel A. The curves are normalized to a maximum of 1 for easy visualization. AFU, arbitrary fluorescence units.
Autopsy-derived brain tissue from PD patients show reduced proteasomal activity (17). Overexpression of expanded poly(Q) proteins or mutant \( \beta \)-H2A51-synuclein inhibits proteasome activity and pharmacological inhibition of the proteasome enhances toxicity and accumulation of these proteins (9–12, 71, 73–75). The impairment of proteasome function by misfolded cytosolic proteins such as expanded poly(Q) proteins can result in failure of ERAD and consequently ER stress (9, 10, 12). ER stress in turn activates apoptosis pathways involving ASK and caspase-12 (12, 51, 76–78). Blocking these pathways for ER stress-mediated apoptosis attenuates expanded poly(Q)-induced cell death. Our results demonstrate that by promoting degradation of misfolded poly(Q) proteins and preserving proteasome function, parkin is able to reduce the activation of caspase-12, an important effector for ER stress-induced cell death thereby improving cell viability. Thus parkin may be important in stress response pathways for eliminating misfolded proteins and preserving proteasome function. ER stress-induced cell death has been implicated in several neurodegenerative diseases (2, 12, 51, 52). Our results raise the possibility that parkin may play a role in modulating some of these neurodegenerative conditions.

Several adult neurodegenerative diseases including both the poly(Q) expansion diseases and PD are associated with the presence of intracellular protein inclusions. The relationship between Lewy bodies and parkin is currently unclear. Parkin-linked PD is commonly cited to lack Lewy bodies; however, the actual number of autopsies is quite small (fewer than 10 total) and one actually contained Lewy bodies (79–83). Despite the absence of Lewy bodies, accumulation of the candidate sub-

**Fig. 9.** Parkin interacts with Hsp70. A, co-immunoprecipitation of Hsp70 with parkin. HEK293 cells were transfected with GFP-Gln79 and FLAG-tagged parkin or myc-tagged E6AP. Cell lysates were processed for immunoprecipitation with anti-FLAG or anti-myc antibodies and immunoblotted for Hsp70. B, parkin structure-function relationship in Hsp70 interaction. The indicated variants were tested for the ability to co-precipitate Hsp70. Top, Hsp70 and FLAG blots; bottom, schematic of the constructs used and summary of the co-immunoprecipitation results. Symbols: Ubl, ■; proximal RING, wide spaced lines in oval; distal RING, thin spaced lines in oval; IBR. C, Hsp70 enhances interaction of parkin and poly(Q) protein in vitro. HA-tagged Gln84 was translated in vitro using a bacterial system in the presence or absence of Hsp70. Hsp70-parkin was expressed in E. coli, purified with TALON resin, and incubated with the translation products. Hsp70-parkin was then immunoprecipitated and HA-Gln84 detected with anti-HA antibody. In a reciprocal experiment, HA-Gln84 was immunoprecipitated and Hsp70-parkin detected with an anti-parkin antibody. D, Hsp70 enhances ubiquitination of Gln79 by parkin. 35S-Labeled ataxin-3 Gln79 was prepared by in vitro translation in bacteria lysate and used for ubiquitination assay with GST-parkin with or without Hsp70 added; asterisk indicates mono-ubiquitinated Gln79.

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strates of parkin (αSp22 and a detergent-insoluble form of Pael-R) has been demonstrated in autosomal recessive juvenile Parkinsonism brains, consistent with impaired protein degradation (2, 4). These observations show that accumulation of Parkinsonism brains, consistent with impaired protein degradation (2, 4). These observations show that accumulation of these aberrant proteins. Cellular models and transgenic Drosophila overexpressing poly(Q)-expanded disease proteins have identified molecular chaperones and other novel genes that modulate poly(Q) expansion diseases (18, 20, 23, 25, 49, 53, 57). Results from these model systems may be relevant to other neurodegenerative diseases, such as PD as evidenced by a recent demonstration that the molecular chaperone Hsp70 also protects against overexpression of human α-synuclein in a transgenic Drosophila model of PD (26). Obviously one does not expect to find poly(Q) aggregates in autosomal recessive juvenile Parkinsonism brains unless the patient also has a genetic mutation involving poly(Q) expansion. Rather we have used poly(Q) proteins as a model to explore mechanisms by which parkin promotes degradation of cytosolic misfolded proteins and reduces cell death. It is not yet known if parkin is a modifier for poly(Q) expansion diseases. Our results suggest that parkin may be a disease modifier for poly(Q) disorders; it would be interesting to test this prediction in transgenic animal models. Although we present results with expanded poly(Q) proteins, we are currently testing if parkin can promote the ubiquitination and degradation of other misfolded proteins involved in neurodegenerative diseases. We have also observed that parkin promotes the degradation of another unrelated misfolded mutant of the coral red fluorescent protein DsRed.

Acknowledgments—We thank Cecile Pickart for helpful advice and critical review of the manuscript, and Kenneth Fischbeck and Henry Paulson for critical review of the manuscript. We have benefited greatly from discussion with Jianxin You and Amy Lam on proteasome binding assays. J. Shi, D. Yarnell, M. Remington, and T. Bowen provided technical assistance for this study.

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Fig. 10. Parkin interacts with the proteasome. A, co-immunoprecipitation of proteasome subunit with parkin. HEK293 cells were transfected with GFP-Gln7 and FLAG-parkin. FLAG-parkin was immunoprecipitated from the cell lysates and proteasome subunit Rpt6/S8 was detected by immunoblotting. For the control, FLAG beads were preincubated with 3×FLAG peptide. B, parkin binds proteasomes directly via its Ubl domain. Left, immobilized bacterially expressed GST-parkin (or GST control) was incubated with purified 26 S proteasome. After washing, bound subunit Rpt6/S8 was detected by immunoblotting. Right panels, pull-down assays were performed using purified 26 S proteasomes and different regions of parkin fused to GST. Bound proteasomes were detected by immunoblotting against Rpt6/S8 (19 S subunit) or HC3 (20 S subunit). C, Coomassie staining of the GST fusion constructs used in panel B.
Parkin Facilitates the Elimination of Expanded Polyglutamine Proteins and Leads to Preservation of Proteasome Function
Yien Che Tsai, Paul S. Fishman, Nitish V. Thakor and George A. Oyler

doi: 10.1074/jbc.M212235200 originally published online April 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212235200

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