Identification of ubiquitin-interacting proteins in purified polyglutamine aggregates

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Abstract Nuclear aggregates of enhanced green fluorescent protein and nuclear localization signal-fused truncated N-terminal huntingtin containing 150 repeats of glutamine residue were purified from ecdysine-inducible mutant neuro2A cell line by sequential extraction of nuclear soluble proteins. To analyze the aggregate-interacting proteins, we subjected the nuclear aggregates to high performance liquid chromatography–mass spectrometry analysis. The resulting data revealed the presence of three new putative aggregate-interacting proteins: ubiquilin 1, ubiquilin 2 and Tollip. These proteins also associated with neuronal intranuclear inclusions in a mouse model of Huntington disease (HD). These aggregate-interacting proteins contain ubiquitin-interacting motifs, suggesting that they are recruited to the aggregates where they may lose their normal function.
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Keywords: Polyglutamine; Aggregate; Ubiquilin; Tollip

1. Introduction

Huntington disease (HD) is a hereditary neurodegenerative disease caused by an expansion of the CAG repeat located in exon 1 of the HD gene [1]. Expansion of the polyglutamine (polyQ) stretch in huntingtin (htt), the HD gene product, leads to the formation of intracellular aggregates [2,3]. Some recent studies demonstrate that nuclear accumulation of insoluble polyQ aggregates or formation of neuronal intranuclear inclusions closely correlate with disease progression [4–6], however it is still unclear whether these aggregates represent a step in pathogenesis or even a protective mechanism.

There are many aggregate-interacting proteins (AIPs), some of which, including the heat shock protein (Hsp) 40, 70 and 90 families, are thought to suppress aggregate formation and cellular toxicity induced by expanded polyQ proteins [7,8]. In addition, functionally important proteins, including transcription factors [9–11] and members of ubiquitin/proteosome pathway [12,13], are sequestrated in the polyQ aggregates, which could cause their loss of function and result in cellular dysfunction. These studies suggest that the components of AIPs reflect either the cellular defense against polyQ aggregates or the cellular machinery affected by polyQ aggregates. Therefore, identification of AIPs should help elucidate the process of aggregate formation, the cellular response to aggregates, and the mechanisms of cellular dysfunction caused by the aggregates. Indeed, we have shown that components of cytoplasmic polyQ aggregates can suppress aggregate formation [8].

In this report, we identified components of nuclear polyQ AIPs from Neuro2a cells stably transfected with tNhtt-150Q-EGFP-NLS, a cDNA encoding htt exon 1 containing 150 CAG repeats and fused with enhanced green fluorescent protein (EGFP) and nuclear translocation signals. Expression of the protein by ecdysone induction caused the formation of nuclear polyQ aggregates [14]. Using this cell model, we have established a method for isolating nuclear polyQ aggregates, which we analyzed by mass spectrometry (MS). These studies identified new AIPs, including ubiquilin 1 and ubiquilin 2, which contain the ubiquitin-associated (UBA) domain, and Toll interacting protein (Tollip), which contains the coupling of ubiquitin conjugation to endoplasimic reticulum degradation (CUE) domain. We also show that these ubiquitin-interacting proteins are associated with neuronal intranuclear inclusions in a mouse model of HD.

2. Materials and methods

2.1. Mice

Heterozygous htt exon 1 transgenic male mice of the R6/2 (145 CAG repeats) strain [Jackson code, B6CBA-TgN (HD exon 1) 62] were obtained from Jackson Laboratory (Bar Harbor, ME). R6/2 mice and their age-matched controls at 15 weeks of age were killed and their brains were collected. Mouse experiments were approved by the animal experiment committee of the RIKEN Brain Science Institute.

2.2. Cell culture

Neuro2a cell lines stably transfected with and expressing tNhtt-150Q-EGFP (HD150Q cells) or tNhtt-150Q-EGFP-NLS (HD150Q-NLS cells) were previously established using the ecdysone-inducible mammalian expression system (Invitrogen, Carlsbad, CA) [14,15]. These cells were maintained in Dulbecco's modified Eagle's medium

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Abbreviations: PolyQ, polyglutamine; HD, Huntington disease; htt, huntingtin; AIP, aggregate-interacting protein; EGFP, enhanced green fluorescent protein; MS, mass spectrometry; UBA, ubiquitin-associated; Tollip, Toll interacting protein; CUE, coupling of ubiquitin conjugation to endoplasmic reticulum degradation; UIM, ubiquitin-interacting motif; UBL, ubiquitin-like

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(Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen) at 37 °C in an atmosphere containing 5% CO_2. To differentiate the cells, 5 mM N^6,2′-*O*-dibutyryl cyclic AMP (dbcAMP; Nacalai Tesque, Kyoto, Japan) was added and to induce the expression of the tNhtt-polyQ-EGFP or tNhtt-polyQ-EGFP-NLS proteins, 1 μM ponasterone A (Invitrogen) was added to the medium.

2.3. Plasmids and transfection

The cDNAs for mouse Tollip and ubiquilin 1 were obtained by reverse transcriptase-PCR from the total RNA fraction of HD150Q-NLS cells. The cDNAs for mouse ubiquilin 2 were obtained from the total RNA fraction of adult mouse brain. PCR products were amplified from the cDNAs and were subcloned into the TOPO-pcDNA3.1/V5-His mammalian expression vector (Invitrogen). All constructs were verified by DNA sequencing. Each plasmid was transfected into the cells using LipofectAMINE 2000 (Invitrogen).

2.4. Antibodies

Polyclonal antibodies were generated in rabbits. Anti-ubiquilin 2 antibody, Ab-Ubql-2, was generated against a peptide corresponding to amino acids 2–18 of mouse ubiquilin 2, and the anti-Tollip antibodies, Ab-Tollip-M and Ab-Tollip-C, were generated against peptides corresponding to amino acids 148–163 and 256–274 of mouse Tollip, respectively. An N- or C-terminal cysteine residue was added to each peptide for coupling to keyhole limpet hemocyanin or to SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL). These polyclonal antibodies were affinity purified using the SulfoLink kit (Pierce Biotechnology). Monoclonal anti-htt (MAB5374) and monoclonal anti-ubiquitin (MAB1510) were from Chemicon International (Temecula, CA); monoclonal anti-Hdj-2 (MS-225) was from Neomarkers (Union City, CA); monoclonal anti-GFP (clones 7.1 and 13.1) was from Roche Molecular Biochemicals (Indianapolis, IN); rabbit polyclonal anti-ubiquilin (#662052) was from Calbiochem (La Jolla, CA); rabbit polyclonal anti-ubiquitin (Z0458) was from Dako (Glostrup, Denmark); rabbit polyclonal anti-Hdj-1 (SPA-400) was from StressGen Biotechnologies (Victoria, BC, Canada); monoclonal anti-V5 (R960-25) and horseradish peroxidase (HRP)-conjugated anti-V5 (R961-25) were from Invitrogen; and goat polyclonal anti-Hsc 70 (SC-1059) and goat polyclonal anti-lamin B (SC-6217) were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG (NA931V) and anti-rabbit IgG (NA934V) were from Amersham Biosciences (Buckinghamshire, UK); and HRP-conjugated anti-goat IgG (#705-035-147) was from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-mouse IgG conjugated with Alexa-546 (A-11030) and anti-rabbit IgG conjugated with Alexa-488 (A-11008) were from Molecular Probes (Eugene, OR).

2.5. Immunocytochemistry and immunohistochemistry

HD150Q-NLS cells growing in 4-well chamber slides were transfected with 0.25 μg of TOPO-pcDNA3.1/V5-His vector containing LacZ, ubiquilin 1, ubiquilin 2 or Tollip. One day after transfection, cells were differentiated and induced to express tNhtt-150Q-EGFP-NLS. After 3 days, the cells were washed twice with cold phosphate buffered saline (PBS), fixed for 20 min in PBS containing 4% paraformaldehyde, washed twice with PBS, permeabilized for 5 min with PBS containing 0.5% Triton X-100, washed several times with PBS, blocked for 1 h with 0.05% Tween 20/Tris-buffered saline (TBST) containing 2% non-fat dried milk, and incubated overnight at 4 °C with primary antibodies (1:1000 for anti-V5 and 1:500 for anti-lamin B). Next, the cells were washed three times with TBST, incubated at room temperature for 1 h with 1:500 Alexa-546 conjugated secondary antibody, and washed three times with TBST. The frozen brains of R6/2 mice mounted in Tissue-Tek were cut into 10 μm sections with a freezing microtome. The sections were washed twice with PBS, fixed for 30 min with 100% methanol, washed several times with PBS, blocked for 1 h with TBST containing 2% non-fat dried milk, and then incubated overnight at 4 °C with primary antibodies. The sections were washed three times with TBST and incubated for 1 h with 1:500 Alexa-488 conjugated secondary antibodies, washed three times with TBST, incubated for 2 h with monoclonal 1:1000 anti-ubiquitin or monoclonal 1:500 anti-htt antibody, washed three times with TBST, incubated for 1 h with 1:500 Alexa-546 conjugated secondary antibody, and washed three times with TBST.

2.6. Isolation of nuclear polyQ aggregates

HD150Q-NLS cells were plated in 150-mm dishes and cultured overnight. The medium was supplemented with 5 mM dbcAMP or both with 5 mM dbcAMP and 1 μM ponasterone A. After 3 days, the cells were washed twice with ice-cold PBS and collected by centrifugation at 1000 rpm for 5 min. Nuclei of these cells were isolated as described previously [16,17] with a slight modification. 1×10^8 HD150Q-NLS cells treated with 5 mM dbcAMP (HD150Q-NLS-D cells) or both 5 mM dbcAMP and 1 μM ponasterone A (HD150Q-NLS-D/I cells) were resuspended in 5 ml of 10 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$, 1% NP-40, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete protease inhibitor EDTA-free (Roche Molecular Biochemicals). After 5 min on ice, the same volume of deionized distilled water was added and swelling on ice was performed for an additional 5 min. Cells were sheared by five passages through a 22-gauge needle and nuclei were recovered by centrifugation at $300 \times g$ for 6 min at 4 °C. Collected nuclei were resuspended in Buffer A (10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.25 M sucrose, 2 mM DTT, 1 mM PMSF, and Complete protease inhibitor EDTA-free) containing 1% NP-40, 500 U/ml DNase I (Sigma, Steinheim, Germany) and 15 U/ml RNase A (Nacalai Tesque), mixed for 60 min at 37 °C, and centrifuged at $1500 \times g$ for 5 min. The pellets were resuspended in Buffer A containing 2 M NaCl, mixed for 60 min at 4 °C, and centrifuged at $1500 \times g$ for 5 min. The pellets were resuspended in Buffer A containing 4% sarcosyl (Nacalai Tesque), mixed for 60 min at 4 °C, and centrifuged for 30 min at $186\,000 \times g$. The pellets were further washed with Buffer A containing 4% sarcosyl, centrifuged for 30 min at $186\,000 \times g$, and collected as the final nuclear insoluble fraction.

2.7. Isolation of cytoplasmic polyQ aggregates

EGFP-positive cytoplasmic polyQ aggregates were collected using a fluorescence-activated cell sorter (FACS; Epics Elite ESP, Beckman Coulter, Fullerton, CA) as described previously [8]. Briefly, HD150Q cells were plated in a 150-mm dish and cultured overnight. The medium was supplemented with 5 mM dbcAMP and 1 μM ponasterone A (HD150Q-D/I cells). After two days, the cells were washed twice with PBS and resuspended in PBS containing 10 mM $MgCl_2$, 500 U/ml DNase I, and 1 U/ml RNase A. The cells were homogenized on ice by 30 strokes at 3000 rpm in a Potter-Elvehjem-type homogenizer with a Teflon pestle controlled by a Digital Homogenizer (As One, Osaka, Japan). The homogenate was applied directly to the argon laser-loaded FACS. The conditions for FACS were as described previously [8]. The collected aggregates were washed twice with PBS containing 4% sarcosyl.

2.8. Identification of AIPs by MS

Purified cytoplasmic aggregates from HD150Q-D/I cells and the final nuclear insoluble fraction of HD150Q-NLS-D and HD150Q-NLS-D/I cells were boiled in sample buffer for SDS–PAGE for 5 min and electrophoresed in a 10% polyacrylamide gel. The gels were stained with SYPRO Ruby (Molecular Probes) and bands in the gels were detected on a UV transilluminator. Each visible band in the insoluble fraction of HD150Q-NLS-D/I cells was excised. For the insoluble fraction of HD150Q-NLS-D cells, gel regions were excised at the same molecular weights as the visible bands in the HD150Q-NLS-D/I insoluble fraction. In-gel digestion was carried out as described elsewhere [18] with a slight modification. The excised gel piece was washed several times for 10 min at 37 °C with 500 μl deionized distilled water. SYPRO Ruby was removed from the gel piece by incubation for 10 min at 37 °C in 100 μl of 50 mM NH_4HCO_3, pH 8.5/50%, CH_3CN. The destained gel piece was dehydrated in 50 μl of CH_3CN for 10 min at 37 °C. The gel piece was reduced by adding 50 μl of 0.01 M DTT/100 mM NH_4HCO_3 and incubating for 15 min at 50 °C, and then alkylated by adding 2 μl of 0.25 M iodoacetate/100 mM NH_4HCO_3 and incubating for 15 min at room temperature. After washing once with 50 μl of 100 mM NH_4HCO_3 and once with 50 μl of 50 mM NH_4HCO_3/50% CH_3CN, the gel piece was dried by vacuum centrifugation. The dried gel piece was covered with 12.5 ng/μl trypsin/100 mM NH_4HCO_3 and then incubated overnight at 37 °C. The tryptic peptides were extracted from the gel by successive incubations with: (i) 50 μl of 50% CH_3CN/1% trifluoroacetic acid for 10 min at 37 °C, (ii) 50 μl of 20% HCOOH/25% CH_3CN/15% isopropanol/40% H_2O for 15 min at 37 °C, and (iii) 50 μl of 80% CH_3CN for 2 min at 37 °C. The extracts from each step were combined and dried by vacuum centrifugation. For peptide

mapping, we used LCQ-Deca XP ion trap mass spectrometer with a nano electrospray ionization source (Thermo Electron Corp., Waltham, MA) combined with a reverse-phase capillary column (Cadenza C18, 0.2×50 mm, Microme BioResources, Inc., Auburn, CA) on a Magic 2002 high performance liquid chromatography system (Microme BioResources, Inc.). The dried tryptic peptides were suspended in 40 μl of 0.1% trifluoroacetic acid, of which 10 μl was applied to the liquid chromatography/MS system. The peptide mixture was separated using mobile phases A and B with a linear gradient over 35 min of 5–65% B, followed by a quick shift to 5% B in 1 min and maintenance at 5% B for 4 min at a flow rate of 1 μl/min (mobile phase $A = 2\%$ CH_3CN/0.1% HCOOH and mobile phase $B = 90\%$ CH_3CN/0.1% HCOOH). The separated peptide fragments were sprayed from a 15 μm i.d. (inner diameter) Picotip (New Objectives, Woburn, MA) and ionized under 1.8 kV of ion spray voltage with a capillary temperature of 260 °C. The scanned mass range was 400–2000 *m/z* and the dynamic exclusion mode was activated throughout the scan. The minimum intensity MS peak for tandem MS (MS/MS) analysis was 2.5×10^5 counts. The MS spectra and MS/MS spectra data were collected by Xcalibur software (Matrix Science, London, UK). The data were analyzed for predicting candidate sequence for the AIPs with MASCOT software (Matrix Science) using the public domain protein database (National Center for Biotechnology Information).

2.9. Western blots

Cell lysates, nuclear fractions, and purified aggregates were prepared as described in Sections 2.5 and 2.6. These materials (20 μg/lane cell lysate, 1×10^6 nuclear aggregate particles/lane, or 0.5×10^6 cytoplasmic aggregate particles/lane) were subjected to SDS–PAGE and then electrophoretically transferred to a polyvinylidene fluoride membrane. After blocking the membrane with TBST containing 2% non-fat milk, the membrane was incubated overnight at 4 °C with primary antibodies (1:2000 for anti-GFP and anti-V5-HRP; 1:1000 for anti-Hdj-1, anti-Hdj-2 and anti-Hsc 70; and 1:500 for anti-htt and anti-ubiquilin) in TBST, washed three times with TBST, incubated for 1 h at room temperature with 1:2000 HRP-conjugated secondary antibodies in TBST, and washed three times. Immunoreactive proteins were detected with enhanced chemiluminescence reagents (Amersham Biosciences).

3. Results

3.1. Purification of tNhtt-150Q-EGFP-NLS and tNhtt-150Q-EGFP aggregates

To purify the nuclear polyQ aggregates, we isolated the nuclei of the HD150Q-NLS-D (differentiated) and HD150Q-NLS-D/I (differentiated/induced) cells and performed sequential extractions of soluble nuclear proteins. DNA-associated proteins and DNA can be removed from the nuclei by treatment with DNase I [19], while nuclear matrix components can be removed by incubation with RNase A, followed by 2 M NaCl [20]. Therefore, to isolate insoluble nuclear components, we treated the isolated nuclei with a combination of RNase and DNase, followed by 2 M NaCl. We also removed remaining soluble nuclear proteins from the nuclei with 4% sarcosyl. As shown in Fig. 1A, the nuclear lamina and chromosomal structures were disrupted, and variable sizes of EGFP-positive nuclear aggregates were found in the final nuclear insoluble fraction. SDS–PAGE of the nuclear insoluble fraction of HD150Q-NLS-D/I cells showed many bands (Fig. 1B, right lane), while that of HD150Q-NLS-D cells lacked distinct bands (Fig. 1B middle lane). Thus, most of the nuclear proteins were removed from the nuclei by the sequential treatments of DNase/RNase, NaCl, and sarcosyl.

To confirm the existence of tNhtt-150Q-EGFP-NLS and other proteins previously reported as AIPs in nuclear insoluble fraction, we conducted Western blot analysis of whole cell lysates, nuclear fractions, and nuclear insoluble fractions

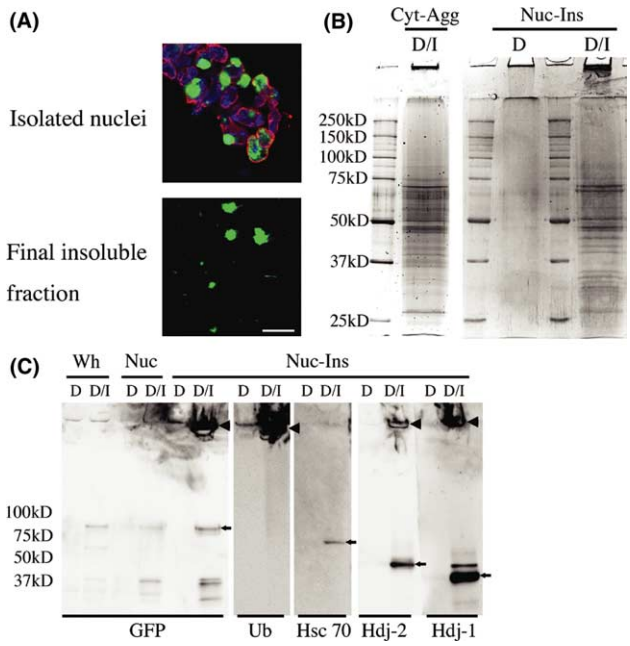

Fig. 1. Purification of nuclear polyQ aggregates. (A) Samples of isolated nuclei and the final insoluble fraction of HD150Q-NLS-D/I cells were processed for immunocytochemistry. The pictures show merged images of EGFP fluorescence, anti-lamin B staining (Alexa-546 as the secondary antibody, red) and chromosome staining with 4′, 6′-diamidino-2-phenylindole, dihydrochloride (DAPI, blue). Bar = 10 μm. (B) SDS–PAGE of purified cytoplasmic aggregates (2.5×10^6) from HD150Q-D/I cells (left lane), nuclear insoluble fraction which contains 5×10^6 nuclear aggregates of HD150Q-NLS-D/I cells (right lane), and the nuclear insoluble fraction of HD150Q-NLS-D from the same amount of nuclei used for the HD150Q-NLS-D/I nuclear insoluble fraction (middle lane). (C) Western blotting for GFP (for tNhtt-150Q-EGFP-NLS), ubiquitin (Ub), Hsc 70, Hdj-1 and Hdj-2 in the insoluble fraction of HD150Q-NLS-D or HD150Q-NLS-D/I cells. Arrow heads indicate gel excluded materials immunoreactive with each antibodies. Arrows indicate each antigenic protein. Ubiquitin immunoreactivity was observed in the gel top and diffuse smearing. Wh, whole cell lysate; Nuc, nuclear fraction; Nuc-Ins, final insoluble fraction of the nuclei.

(Fig. 1C) from HD150Q-NLS-D and D/I cells. Western blotting with an anti-GFP antibody detected an ~80-kDa band in the nuclear insoluble fraction of HD150Q-NLS-D/I cells, corresponding to the tNhtt-150Q-EGFP-NLS protein. This antibody also detected concentrated aggregates at the top of the gel in the nuclear insoluble fraction from HD150Q-NLS-D/I cells. Ubiquitin, Hsc 70, Hdj-1 and Hdj-2 were detected in the nuclear insoluble fraction from HD150Q-NLS-D/I cells.

3.2. Identification of UBA and CUE domain containing proteins in the purified nuclear polyQ aggregates

Following SDS–PAGE of the purified nuclear polyQ aggregates from HD150Q-NLS-D/I cells, each band was excised, digested with trypsin, and subjected to MS/MS analysis. As a negative control, we also excised and analyzed the gel pieces in the SDS–PAGE of the nuclear insoluble fraction from HD150Q-NLS-D cells. We identified several proteins containing UBA or CUE domains (Table 1) in the purified nuclear polyQ aggregates from the HD150Q-NLS-D/I cells. We also found members of the Hsp 70 and 40 families. These proteins were not found in the insoluble fraction of HD150Q-NLS-D cells.

Table 1
AIP candidates suggested by MS/MS analysis

Identified proteins	Nominal mass[a] (Da)	Sequence coverage[b] (%)	GI number[c]	Accession number
Ubiquilin 1	61 976	12	20072434	BC026847.1
Ubiquilin 2	67 379	16	34328236	NM_018798.2
Tollip	30 573	21	13591860	NM_023764.2
Hsc 70	71 021	34	476850	A45935
Hsp 40	45 581	73	6680297	NM_008298.2

[a] Molecular weight of each protein as calculated by MASCOT.
[b] Percentage of amino acids in the full-length protein covered by the identified peptides.
[c] "GenInfo Identifier" sequence identification number for the protein.

3.3. *Ubiquitin-interacting proteins co-localize with polyQ aggregates in the nuclei of HD150Q-NLS cells*

We next used immunocytochemistry to determine whether these UBA and CUE domain-containing proteins co-localize with polyQ aggregates. The cDNA of mouse ubiquilin 1, ubiquilin 2 and Tollip were cloned into the TOPO-pcDNA3.1/V5-His mammalian expression vector, after which the constructs were transfected into HD150Q-NLS cells. Fig. 2 shows that the V5-tagged AIP candidates co-localized with nuclear polyQ aggregates. Although overexpressed ubiquilin 1, ubiquilin 2 and Tollip accumulated in the perinuclear regions of the cells (data not shown), we confirmed that ubiquilin 1, ubiquilin 2 and Tollip co-localized with nuclear polyQ aggregates in some cells (co-localization ratio = the number of cells having V5-positive aggregates/the number of transfected cells having aggregates: LacZ, 0%; ubiquilin 1, 13%; ubiquilin 2, 18%; Tollip, 35% in HD150Q-NLS-D/I cells and; LacZ, 3.7%; ubiquilin 1, 61%; ubiquilin 2, 79%; Tollip, 83% in HD150Q-D/I cells.)

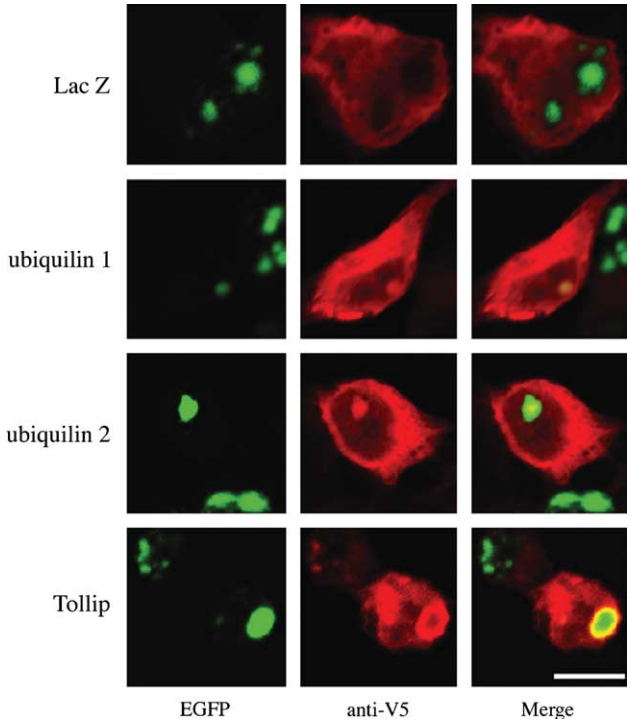

Fig. 2. Ubiquitin-interacting proteins are associated with polyQ aggregates. Immunocytochemistry of HD150Q-NLS cells transfected with Lac Z-V5-His, ubiquilin 1-V5-His, ubiquilin 2-V5-His and Tollip-V5-His. Each overexpressed protein is labeled by anti-V5 antibody (Alexa fluor 546 as the secondary antibody, red). Ubiquilin 1-V5-His, ubiquilin 2-V5-His and Tollip-V5-His co-localized with nuclear polyQ aggregates of the HD150Q-NLS cells. Bar = 10 μm.

3.4. *Ubiquitin-interacting proteins exist in purified cytoplasmic and nuclear polyQ aggregates*

We performed Western blotting to verify the presence of these proteins in the aggregates. To this end, we generated polyclonal antibodies Ab-Ubql-2 against ubiquilin 2 and Ab-Tollip-M and Ab-Tollip-C against Tollip (Fig. 3A). We also used a commercially available rabbit polyclonal anti-ubiquilin

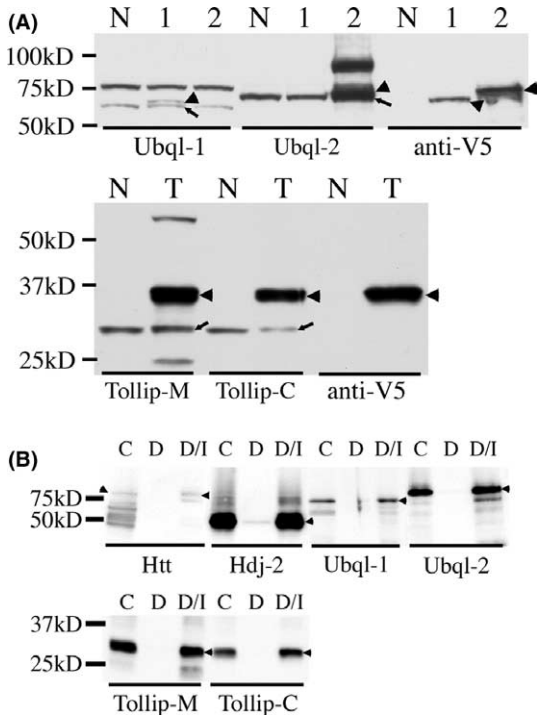

Fig. 3. Ubiquitin-interacting proteins exist in purified cytoplasmic and nuclear aggregates. (A) Characterization of anti-ubiquilin (Ab-Ubql-1), Ab-Ubql-2, Ab-Tollip-M, and Ab-Tollip-C antibodies. Ab-Ubql-1 recognizes endogenous and overexpressed ubiquilin 1 and an ~80 kDa non-specific band. Ab-Ubql-2 recognizes endogenous and overexpressed ubiquilin 2 and its ~90 kDa derivative. Ab-Tollip-M and -C recognize endogenous and overexpressed Tollip. Ab-Tollip-M also detects its derivatives. Cell lysates from HD150Q-NLS cells transfected with; N-lane, no vector; 1-lane, ubiquilin 1-V5-His expressing vector; 2-lane, ubiquilin 2-V5-His expressing vector; or T-lane, Tollip-V5-His expressing vector. Arrows indicate endogenous protein and arrow heads indicate overexpressed protein. (B) Ubiquilins 1, ubiquilin 2 and Tollip coexist in purified cytoplasmic and nuclear poly Q aggregates. C-lane, purified cytoplasmic aggregates of HD150Q-D/I cells; D-lane, nuclear insoluble fraction of HD150Q-NLS-D cells; D/I-lane, purified nuclear aggregates of HD150Q-NLS-D/I cells.

antibody (Ab-Ubql-1) to identify ubiquilin 1 (Fig. 3A). Western blot analysis confirmed that ubiquilin 1, ubiquilin 2 and Tollip co-purify with nuclear polyQ aggregates (Fig. 3B). These proteins were also recognized in purified cytoplasmic aggregates fraction (Fig. 3B). These findings suggested that ubiquilin 1, ubiquilin 2 and Tollip are strongly associated with polyQ aggregates and shift to the insoluble fraction upon formation of polyQ aggregates.

We also used anti-GFP immunoprecipitation to determine whether the ubiquitin-interacting proteins associate with soluble tNhtt-150Q-EGFP-NLS in the HD150Q-NLS cells. We found that Hdj-2 but not soluble Tollip or ubiquilin 1 and 2 co-immunoprecipitate with tNhtt-150Q-EGFP-NLS (data not shown). Thus, Tollip and ubiquilin 1 and 2 interact specifically with the insoluble and highly ubiquitinated form of tNhtt-150Q-EGFP-NLS.

3.5. *Tollip and ubiquilin 1 and 2 co-localize with neuronal nuclear inclusions in a transgenic mouse model of HD*

Our results suggested that ubiquilin 1, ubiquilin 2 and Tollip are AIPs. To investigate this further, we examined whether these proteins associate with polyQ aggregates in HD exon 1 transgenic mice (R6/2 mice) [21]. Immunostaining of R6/2 transgenic mouse brain sections showed that ubiquilin 1, ubiquilin 2 and Tollip co-localize with ubiquitin and htt immunoreactive neuronal intranuclear inclusions, while immunostaining of age-matched control mouse brain sections showed that ubiquilin 1, ubiquilin 2 and Tollip did not form intranuclear inclusions (Fig. 4). We also counted two hundred htt-positive neuronal intranuclear inclusions both in the cortex and striatum of R6/2 transgenic mouse. We confirmed that all of the inclusions associated with ubiquilin 1, ubiquilin 2 or Tollip. These findings indicate that our method for purifying nuclear polyQ aggregates from a cell-based model can identify AIPs associated with a mouse model of HD.

4. Discussion

In this study, we established a method for purifying nuclear polyQ aggregates from a cell model of HD. There have been a few attempts to purify the polyQ aggregates and to directly identify their components [8,22,23]. In one of these studies, polyQ aggregates were isolated by detergent insolubility and density gradient fractionation [22], while other studies collected aggregates depending on GFP fluorescence by using FACS [8,23]. These methods successfully isolated uniform and particle-like aggregates. In the current studies, we purified the nuclear aggregates by sequential removal of soluble proteins. This method was intended to identify the nuclear proteins that are soluble under normal conditions but shift to the insoluble upon formation of polyQ aggregates. The advantage of our method is that a broad range of aggregate sizes can be isolated regardless of the intensity of the GFP fluorescence. These may include aggregates in a variety of stages of formation. Moreover, using this method, we identified not only a group of proteins that were already known as AIPs, including members of the Hsp 40 and 70 families, but also several ubiquitin-interacting domain-containing proteins as new candidate AIPs.

UBA domain, ubiquitin-interacting motif (UIM), and CUE domain are known to bind directly to mono- and/or polyUb chains [24,25]. PolyQ aggregates have been reported to contain several UBA domain-containing proteins, including p62, HHR 23A, B and A1Up, as well as UIM domain-containing proteins, such as proteosome subunit S5a and ataxin 3 [26–28]. These ubiquitin-interacting proteins are thought to be sequestered in the polyQ aggregates through these domains, and loss of function of these proteins might be the cause of neurodegeneration [26]. In this study, we showed that the UBA domain-containing proteins, ubiquilin 1 and 2, and the CUE domain-containing protein, Tollip, are associated with polyQ aggregates in both cellular and mouse models of HD. These

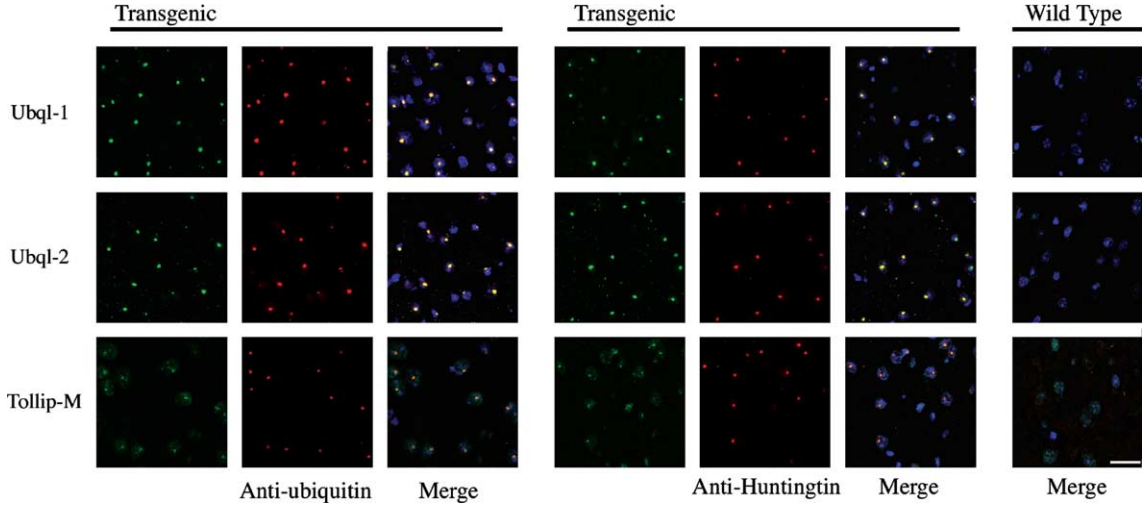

Fig. 4. Ubiquitin-binding proteins are associated with neuronal intranuclear inclusions. Frozen sections of cerebral cortex prepared from R6/2 transgenic and wild type mice were double-labeled with a monoclonal anti-ubiquitin or a monoclonal anti-htt antibody (Alexa-546, red) and Ab-Ubql-1, Ab-Ubql-2, or Ab-Tollip-M antibodies (Alexa-488, green), followed by staining with DAPI (blue). As for wild type, only merged image of cerebral cortex stained with each AIP antibody, anti-htt and DAPI was shown. Ubiquilins 1, ubiquilin 2 and Tollip co-localized with neuronal intranuclear inclusions of transgenic mouse. Bar = 20 μm.

proteins had not been previously reported to associate with polyQ aggregates.

Ubiquilins, also known as plics, are members of the proteins, which possess an N-terminal ubiquitin-like (UBL) domain and a C-terminal UBA domain, and are closely related to A1Up, which interacts with ataxin 1 [28]. The UBL domain interacts with subunit of the 19S complex and UBA domains interact with K48-linked polyubiquitin chains [29,30]. Overexpression of ubiquilins interferes with ubiquitin/proteosome-dependent degradation of p53 and IκBα [31]. In addition, disruption of *DSK2*, a yeast homolog of ubiquilin, inhibits the degradation of a model substrate, and overexpression of Dsk2 protein causes the accumulation of polyubiquitin protein [29]. Thus, ubiquilins are thought to modulate the ubiquitin/proteosome pathway. Ubiquilin 1 is also known to stabilize intracellular $GABA_A$ receptor subunits and to increase the number of receptors in the plasma membrane [32], suggesting that ubiquilin 1 has a crucial role in the neuron. Furthermore, ubiquilin 1 colocalizes with neurofibrillary tangles in Alzheimer disease and with Lewy Bodies in Parkinson disease [33], indicating that ubiquilin may play a role in neurodegenerative diseases.

Tollip was initially identified as a modulator of interleukin-1 signaling [34]. This protein has an N-terminal protein kinase C conserved region 2 (C2 domain) and a C-terminal CUE domain. A recent study revealed that Tom 1, a VHS (Vps27, Hrs and STAM) domain-containing protein, interacts with the N-terminal region of Tollip, polyubiquitin chains, and clathrin heavy chain, and that Tollip binds to polyubiquitin chains, suggesting that the Tom 1-Tollip complex can link polyubiquitinated proteins to clathrin [35]. Therefore, sequestration of ubiquilins or Tollip in the polyQ aggregates may disrupt normal neuronal function by altering the ubiquitin/proteosome pathway or clathrin-mediated vesicular trafficking.

A variety of methods, including yeast two-hybrid screening [36] and genetic screening in yeast [37] or *Drosophila* [38], have been utilized to identify polyQ disease-related proteins that may mediate the neurodegenerative phenotypes. The method employed in this report should also help identify molecules in the polyQ disease cascade and may provide further information on the process of polyQ aggregation and its role in neurotoxicity.

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