

Sequestration of Glyceraldehyde-3-phosphate Dehydrogenase to Aggregates Formed by Mutant Huntingtin

Junchao WU, Fang LIN, and Zhenghong QIN*

Department of Pharmacology and Laboratory of Aging and Nervous Diseases, Soochow University School of Medicine, Suzhou 215123, China

Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported to interact with proteins containing the polyglutamine (polyQ) domain. The present study was undertaken to evaluate the potential contributions of the polyQ and polyproline (polyP) domains to the co-localization of mutant huntingtin (htt) and GAPDH. Overexpression of N-terminal htt (1–969 amino acids) with 100Q and 46Q (htt1-969-100Q and htt1-969-46Q, mutant htt) in human mammary gland carcinoma MCF-7 cells formed more htt aggregates than that of htt1-969-18Q (wild-type htt). The co-localization of GAPDH with htt aggregates was found in the cells expressing mutant but not wild-type htt. Deletion of the polyP region in the N-terminal htt had no effect on the co-localization of GAPDH and mutant htt aggregates. These results suggest that the polyQ domain, but not the polyP domain, plays a role in the sequestration of GAPDH to aggregates by mutant htt. This effect might contribute to the dysfunction of neurons caused by mutant htt in Huntington's disease.

Keywords huntingtin; GAPDH; polyglutamine; polyproline

Huntington's disease (HD) is an adult-onset, autosomal-dominant neurodegenerative disorder. Patients with HD are characterized by hyperkinetic involuntary movement, cognitive impairment, and depression [1]. The gene responsible for HD was identified as *IT15*. This gene contains a CAG repeat that encodes the polyglutamine (polyQ) tract in the N-terminus of huntingtin (htt). This trinucleotide repeat is highly polymorphic. The normal range of the repeat is from 10 to 35Q. The disease-causing mutation was identified as an expansion of this repeat to more than 36 triplets [2]. In a variety of HD animal models, cellular accumulation of mutant htt (mhtt) is a critical step to HD pathology [3–6]. The polyproline (polyP) domain is a proline-rich region located immediately after polyQ. It is an important domain for many protein-protein interactions. It has been reported that polyP interacts with proteins containing WW domains including huntingtin-protein interactors, htt yeast partners (HYPs; HYPA, HYPB and

HYPC), that appear to have a higher affinity for expanded polyQ htt [7].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) has been well studied as a classical glycolytic protein in energy production. In fact, GAPDH is a multifunctional protein and plays roles in a variety of activities, including endocytosis and membrane fusion, microtubule bundling and translational regulation, nuclear transfer RNA export, as well as DNA replication and DNA repair [8]. *In vivo*, GAPDH has three forms: tetramer of identical 37 kDa subunits; dimer; and monomer. These forms are related to its diverse functions. Its glycolytic activity is restricted to the tetramer, whereas the dimeric and monomeric structures are required for its other functions.

In the last 10 years, the contribution of GAPDH to neurodegenerative diseases has been reported [9–11]. Some *in vitro* studies have shown an interaction between GAPDH and the mutated forms of several CAG trinucleotide repeat-containing proteins. It was thought to be the causative factor in certain neurodegenerative diseases [12,13]. The interaction between htt and GAPDH was proposed as one possible cause of cell death in HD [8]. As

Received: April 18, 2007 Accepted: June 20, 2007

This work was partially supported by the grants from the National Natural Science Foundation of China (No. 30370506) and the Specialized Research Fund for the Doctoral Program of Higher Education, China (No. 20050285017)

*Corresponding author: Tel, 86-512-65880406; Fax, 86-512-65880406; E-mail, zhqin5@hotmail.com

DOI: 10.1111/j.1745-7270.2007.00352.x

polyQ and polyP are important domains in htt and are involved in many protein interactions [14], this study was designed to investigate whether the polyQ and polyP domains took part in the interactions between GAPDH and htt.

Materials and Methods

Materials

Huntingtin 1-969 expression plasmids, including pcDNA3-htt1-969-18Q, pcDNA3-htt1-969-46Q, pcDNA3-htt1-969-46Q with proline deletion, and pcDNA3-htt1-969-100Q, were kindly provided by Dr. Marian DiFIGLIA (Massachusetts General Hospital and Harvard Medical School, Boston, USA). These constructs encode an N-terminal htt fragment (1–969 amino acids) with 18, 46, or 100Q and a Flag tag. To study the role of polyP in the interaction between htt and GAPDH, a polyP rich region was deleted from htt1-969-46Q. Human mammary gland carcinoma cell line MCF-7 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences (Shanghai, China). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Company (Hangzhou, China). The transfection reagent SuperFect was purchased from Qiagen (Valencia, USA).

Cell culture and transient transfection

MCF-7 cells are epithelial-type cells derived from human mammary gland carcinoma. Cells were cultured in 25 cm² flasks in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 mg/L streptomycin, and 100 U/ml penicillin, and incubated in a humidified atmosphere of 5% CO₂ at 37 °C. SuperFect was used for transfection. At 80% confluence, cells were incubated in 2 ml RPMI 1640 containing 5–10 µg htt cDNA and 36 µl SuperFect. After incubation for 3 h, cells were given 3 ml fresh RPMI 1640 medium, and culturing was continued to the desired time.

Protein preparation and Western blot analysis

For preparation of whole cell lysates, cells were scraped off the flasks and centrifuged at 500 g for 5 min, 24 h after transfection. The cell pellets were rinsed with ice-cold phosphate buffer solution (PBS) twice. Five volumes of Western blot lysing buffer (containing 20 mM Tris-HCl, pH 7.0, 20 mM EDTA, 5% Triton X-100 supplemented with a cocktail of protease inhibitors containing

1 mM pefablock, 100 U/ml aprotinin, and 10 µg/ml leupeptin) for each volume of cell pellets was added, then the mixture was incubated on ice for 10 min and sonicated on ice (1 s/ml per sonicate, an interval of 30 s, a total of five times). The lysate was microcentrifuged at 10,600 g at 4 °C for 10 min and supernatant was preserved at –70 °C for later use. Protein concentration was determined with a BCA kit (Pierce, Rockford, USA). Samples were mixed with loading buffer and boiled for 5 min. An aliquot of 30 µg protein from each sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel, and protein was subsequently transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20, and immunoblotted with anti-GAPDH monoclonal antibody (1:1000 dilution; Advanced Immunochemical, Long Beach, USA) at 4 °C overnight. Excess primary antibody was removed by three washes with 0.1% Tween-20. The reaction of primary antibodies was detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) with a dilution of 1:5000 in blocking solution for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, USA) and visualized by autoradiography. Actin was used as a loading control and detected with a mouse monoclonal antibody (Sigma, St. Louis, USA).

Immunofluorescence

MCF-7 cells were cultured on poly-L-lysine-coated microslips and transfected with different cDNAs encoding htt proteins as described above. Cells were washed with PBS and fixed with 4% paraformaldehyde (Fluka, Buchs, Schweiz) in PBS for 15 min at room temperature. Cells were subsequently washed with PBS and incubated in PBS containing 0.1% Triton X-100 for 10 min. After being washed again with PBS, the cells were then incubated for 1 h in PBS containing 2% non-fat milk (Bright Dairy and Food, Shanghai, China) at room temperature. Cells were then incubated overnight at 4 °C in a blocking solution containing rabbit anti-htt polyclonal antibody Ab1 (gift from Dr. Marian DiFIGLIA) with a dilution of 1:1500 and mouse anti-GAPDH monoclonal antibody (Immunochemical) with a dilution of 1:1000. Cells were then incubated with fluorescein-isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G antibody with a dilution of 1:400 and Cy3-conjugated donkey anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch Laboratories) with a dilution of 1:600 for 2 h at room temperature [15].

Immunostained cells were examined with a confocal microscope (Radiance 2001; Bio-Rad, Hercules, USA) using a $\times 100$ oil immersion lens. Confocal images were captured with Laser Sharp 2000 software and merged in Adobe Photoshop (Version 8.0). To quantify the co-localization of GAPDH and htt aggregates in the cells expressing htt, 100 htt aggregate-positive cells per microslip were randomly scanned with a confocal microscope, and six microslips were used for each group. The examiner was unaware of the experimental conditions.

Statistics analysis

Statistical analysis was carried out with one-way ANOVA. $P < 0.05$ was considered to be significant.

Results

Expression of GAPDH in MCF-7 cells after transfection of htt

The level of GAPDH in MCF-7 cells after expression of htt was determined by Western blot analysis. GAPDH was detected as a major protein band, using immunoblotting, with the expected molecular weight. The level of GAPDH was not significantly altered by expressed htt (**Fig. 1**; $P > 0.05$).

Co-localization of mhtt and GAPDH

Twenty-four hours after transfection of htt1-969-18Q, htt1-969-100Q and htt1-969-46Q, double immunofluores-

cence of htt and GAPDH was used for identification of the expression of the two proteins in MCF-7 cells. As shown in the confocal images, htt aggregates were observed in the cytoplasm in the cells expressing htt1-969-18Q, htt1-969-100Q or htt1-969-46Q. GAPDH in control cells [**Fig. 2(A)**, top panel] and cells expressing htt1-969-18Q [**Fig. 2(A)**] appeared dispersed in the cytoplasm. No co-localization of high levels of GAPDH in htt aggregates was formed by htt1-969-18Q. In contrast, immunofluorescence patches of GAPDH were found in the cells expressing htt1-969-100Q and htt1-969-46Q. These GAPDH patches were co-localized with htt aggregates [**Fig. 2(A)**]. Quantitative analysis of htt-positive cells bearing mhtt aggregates revealed that sequestration of GAPDH was found in approximately 60% of these cells. There was no sequestration of GAPDH by htt aggregates formed by wild-type htt [**Fig. 2(B)**].

Influence of polyP domain on interaction between mhtt and GAPDH

Double immunofluorescence was used to identify the expression of GAPDH and mhtt proteins in MCF-7 cells transfected with htt1-969-46Q and htt1-969-46Q with polyP deletion. The results showed that GAPDH localized to htt aggregates in the cells transfected with either htt1-969-46Q or htt1-969-46Q with polyP deletion [**Fig. 3(A)**]. Quantitative analysis of htt-positive cells bearing mhtt aggregates revealed that sequestration of GAPDH was not altered in the cells expressing htt1-969-46Q with or without polyP deletion [**Fig. 3(B)**; $P > 0.05$].

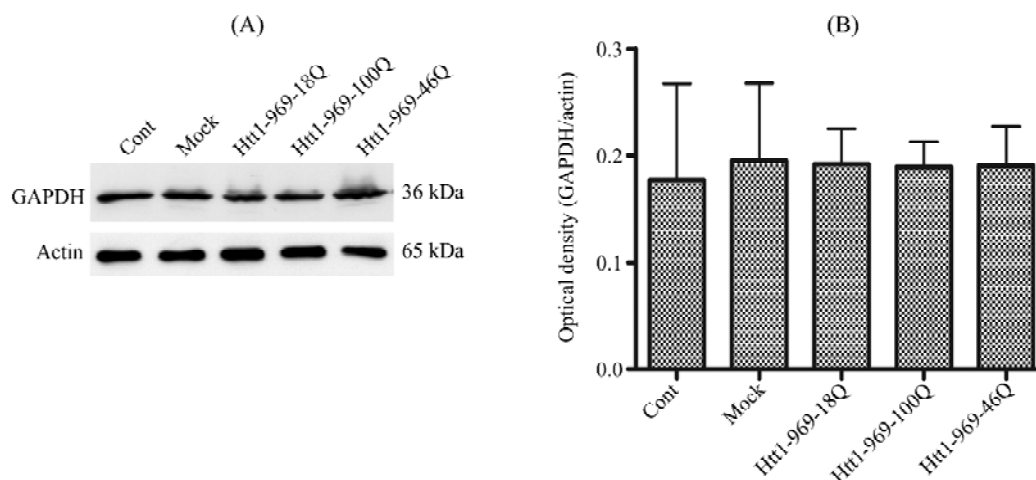


Fig. 1 Western blot analysis of GAPDH in human mammary gland carcinoma MCF-7 cells

MCF-7 cells were transfected with wild-type htt (htt1-969-18Q) or mutant htt (htt1-969-100Q, htt1-969-46Q). Cells were harvested and processed for immunoblotting 24 h after transfection. Cont, control cells that had no special treatment; mock, cells treated only with transfection reagent.

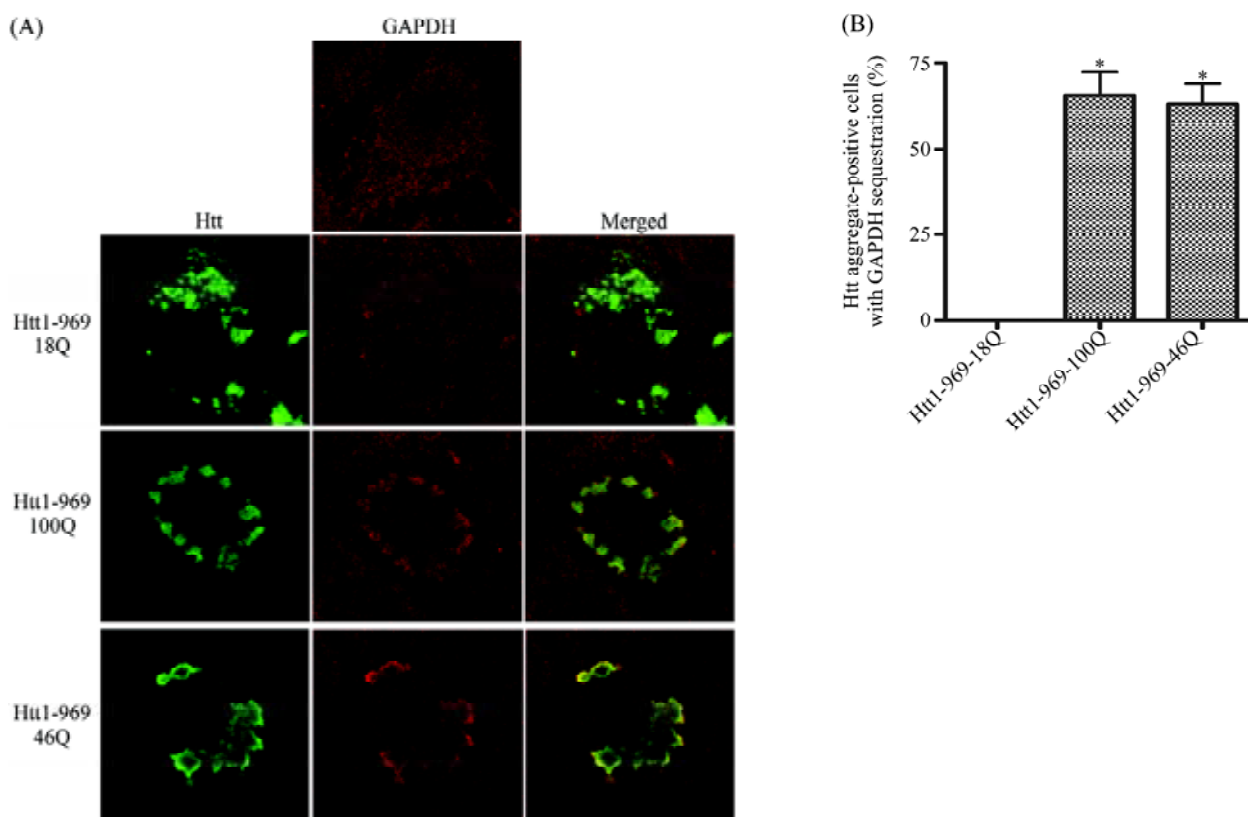


Fig. 2 Co-localization of mutant huntingtin (mhtt) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human mammary gland carcinoma MCF-7 cells

MCF-7 cells were transfected with mild-type htt (htt1-969-18Q) or mutant htt (htt1-969-100Q, htt1-969-46Q). Cells were fixed with 4% paraformaldehyde and processed for immunofluorescence 24 h after transfection. Cells were analyzed using a confocal microscope ($\times 1000$). (A) Co-localization of mhtt and GAPDH was indicated by color change to yellow. (B) Quantitative analysis of co-localization of GAPDH and htt aggregates. $n=6$. $*P<0.05$.

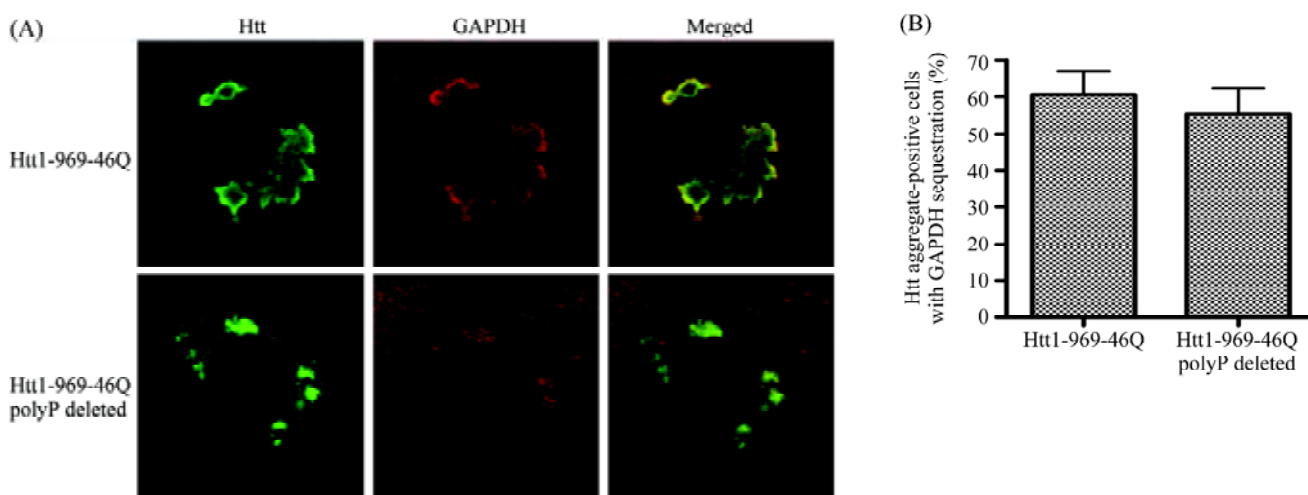


Fig. 3 Co-localization of mutant huntingtin (htt) cDNA constructs, htt1-969-46Q or htt1-969-46Q with polyproline (polyP) deletion, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Human mammary gland carcinoma MCF-7 cells were transfected with htt1-969-46Q or htt1-969-46Q with polyP deletion. Cells were fixed with 4% paraformaldehyde and processed for immunofluorescence 24 h after transfection. Cells were analyzed with a confocal microscope ($\times 1000$). (A) Co-localization of mutant huntingtin (htt) and GAPDH is indicated by color change to yellow. (B) Quantitative analysis of co-localization of GAPDH and htt aggregates. $n=6$. $P>0.05$.

Discussion

Many studies have found that mhtt obtained abnormal interactions with other cytoplasmic and nuclear proteins [16–19]. Recruitment or sequestration of cellular proteins might be possible causes of cell death in HD [20]. Htt interacts with a number of proteins including GAPDH, a key glycolytic enzyme in the process of glycolysis, and such binding was relevant to the nuclear targeting and cytotoxicity of mhtt [21].

The present study confirmed the domains of htt involved in its interaction with GAPDH. Four constructs of htt cDNA were used, one was wild-type htt1-969-18Q and the other three were mutant types, htt1-969-100Q, htt1-969-46Q, and htt1-969-46Q with polyP deletion. Using Western blot analysis, we found that expression of either wild-type (wt) or mutant htt did not change the overall levels of GAPDH. However, GAPDH formed patches and localized to htt aggregates in the cells expressing mhtt. In contrast, expression of wt htt had no effect on cellular distribution of GAPDH. This suggests that GAPDH might be sequestered by mhtt into htt aggregates. Our previous research revealed that htt was cleaved by several proteases, such as caspases and calpains [22]. The cleavage of htt by these proteases produces N-terminal htt fragments. N-terminal htt is prone to aggregate in cells and might be the cause of neuronal death in HD. It is critical for cytotoxicity induced by HD. In our previous studies, we found that htt bodies formed either by wt htt or mhtt sequestered many cytoplasmic proteins in a polyP-dependent manner [14]. The present study revealed that aggregates formed by mhtt, but not wt htt, could sequester GAPDH, suggesting that this kind of sequestration is dependent on expanded CAG repeats. We also examined the relationship of GAPDH and the polyP domain in htt. We found that GAPDH still colocalized with mhtt aggregates in the absence of the polyP domain. This suggests that sequestration of GAPDH by mhtt is dependent on an expanded polyQ, but not polyP domain. The sequestration of GAPDH by mhtt might affect cell viability as GAPDH has important cellular functions.

In fact, many proteins with CAG expansions bind to GAPDH. mhtt-GAPDH interactions through this expanded polyQ domain could not only affect energy production but also result in pleiotropic effects involving various biochemical pathways in HD cells. Other *in vitro* studies have also shown that GAPDH glycolytic activity was specifically reduced in Alzheimer's disease and HD [23]. Furthermore, mitochondria dysfunction and impairment of energy metabolism have long been implicated in HD

pathogenesis [24]. There are several reports suggesting that interactions between mhtt and GAPDH might play important roles in HD pathogenesis. This notion is supported by data from HD patients showing reduced ATP production and increased lactate production [25], and supported by animal studies showing that compromising mitochondria function produced striatal lesions mimicking human HD pathology [26]. In HD fibroblasts, reduced GAPDH activity has been reported in subcellular fractions [10]. Additionally, the ability of GAPDH to translocate to the nucleus might also be related to apoptosis of cells [27], causing neuronal cell death in HD. GAPDH might act as a chaperon through its selective binding to the polyQ region of htt. Senatorov *et al.* also reported that GAPDH was directly or indirectly involved in the initiation of apoptotic cascades in a transgenic mouse model of HD [28]. Mazzola and Sirover identified an abnormal nuclear GAPDH structure in HD, a high molecular weight species that alters GAPDH function [10]. Nuclear GAPDH will interact with DNA to affect nuclear transfer RNA transport, DNA replication, and DNA repair [29], and to modulate calcium release [30]. All of these lines of evidence suggest that interactions between mhtt and GAPDH could play a role in cell death in HD. The present results showed that GAPDH could be sequestered to htt aggregates formed by mhtt, providing additional evidence in support of the notion that mhtt might have an effect on physiological functions of GAPDH. However, the exact function of GAPDH in the pathogenesis of HD remains unclear. Further studies are warranted to examine the sequestration of GAPDH in HD patients and the functional impact of GAPDH sequestration in the cells expressing mhtt.

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Edited by
Guanghui WANG