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Expression of expanded polyglutamine targets profilin for degradation and alters actin dynamics

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Huntington's disease is caused by polyglutamine expansion in the huntingtin protein. Huntingtin directly interacts with profilin, a major actin monomer sequestering protein and a key integrator of signals leading to actin polymerization. We observed a progressive loss of profilin in the cerebral cortex of Huntington's disease patients, and in cell culture and *Drosophila* models of polyglutamine disease. This loss of profilin is likely due to increased degradation through the ubiquitin proteasome system. Profilin loss reduces the F/G actin ratio, indicating a shift in actin polymerization. Overexpression of profilin abolishes mutant huntingtin toxicity in cells and partially ameliorates the morphological and functional eye phenotype and extends lifespan in a transgenic polyglutamine *Drosophila* model. These results indicate a link between huntingtin and profilin and implicate profilin in Huntington's disease pathogenesis.

Keywords: Profilin; Huntington's disease; Polyglutamine; Actin; Ubiquitin proteasome system

Introduction

Huntington's disease (HD) is an autosomal dominantly inherited progressive neurological disease that belongs to a group of neurodegenerative disorders caused by expansion of polyglutamine tracts in otherwise unrelated proteins (Zoghbi and Orr, 2000). Expansion of a $(CAG)_n$ trinucleotide repeat within the first exon of the *IT15* gene leads to an abnormally long polyglutamine tract in the huntingtin (htt) protein (The Huntington's Disease Collaborative Research Group, 1993). Polyglutamine expansion increases the propensity of htt to aggregate and causes neuronal degeneration, particularly in the caudate nucleus of the basal ganglia and in the cerebral cortex. Htt is a 350 kDa, ubiquitously expressed protein containing a proline-rich region adjacent to the polyglutamine tract in its N-terminus as well as numerous HEAT repeats throughout the protein. Although its cellular function remains unclear, htt has been implicated in several processes, including cell signalling, endocytosis, and vesicle transport, based on the known functions of its interacting partners (Harjes and Wanker, 2003). The underlying molecular mechanism of HD pathogenesis is yet to be fully elucidated, but it is thought that mutant htt gains novel toxic properties, possibly through polyglutamine dependent conformational changes and aberrant protein interactions.

Various htt interactors have been identified through yeast two-hybrid screening, co-immunoprecipitation, and *in vitro* binding assays, including proteins that are regulators of cytoskeletal organization and participants in endocytic pathways (Wanker et al., 1997; Singaraja et al., 2002; Goehler et al., 2004). One of the proteins thus identified is profilin-2a (Goehler et al., 2004). Profilins are eukaryotic actin monomer (G actin) binding proteins that interact with proline-rich regions in a variety of proteins (reviewed by Witke (2004)) and regulate actin dynamics. Profilin was originally thought to be a G actin sequestration protein that inhibits actin polymerization, but subsequent studies have shown that, depending on the conditions, profilin may either sequester G actin or promote actin polymerization (Pantaloni and Carlier, 1993).

In mammals, there are four profilin genes, with tissue-specific expression. Profilin-1 is the major isoform in most tissues; profilin-2 is predominantly expressed in the brain (Kwiatkowski and Bruns, 1988); and profilins 3 and 4 are almost exclusively expressed in the testis (Hu et al., 2001; Obermann et al., 2005). Profilin-2 has two splice variants, 2a and 2b (Di Nardo et al., 2000; Lambrechts et al., 2000), and shares approximately 60% sequence identity with profilin-1. Neurons express profilin-1 and -2a (Lambrechts et al., 2000; Neuhoff et al., 2005), and biochemical studies have suggested that both isoforms are closely related with respect to actin binding and their affinity for poly(L-proline). Ablation of profilin-1 expression in knockout mice results in early embryonic lethality (Witke et al., 2001). Mice lacking

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profilin-2, although viable, show abnormalities in neurotransmitter release and presynaptic excitability, highlighting the importance of profilin-2 in the central nervous system (Pilo Boyl et al., 2007).

Here we report that profilin levels are progressively reduced in cortical tissue from HD patients, in cultured cells expressing mutant htt, and in a *Drosophila* polyglutamine model. We further demonstrate that restoration of profilin mitigates polyglutamine-induced toxicity in cells and in *Drosophila*, indicating that profilin may be involved in the HD pathogenesis.

Materials and methods

Antibodies

Polyclonal antibodies against profilin-1 and -2a were generated by Open Biosystems (Huntsville, AL) by injecting into rabbits the linear peptides KCYEMASHLRRSQY and KAYSMAKYLRDSGF conjugated to KLH. Following immunization, blood was collected, and crude serum was purified with an affinity column containing the respective profilin peptide. Other antibodies used include a monoclonal anti-chickadee, chi1J (Developmental Studies Hybridoma Bank, University of Iowa), monoclonal anti-actin (Chemicon), monoclonal anti-tubulin (Chemicon), monoclonal anti-actin (AC-40; Sigma-Aldrich), monoclonal anti-GFP (BD Biosciences), and rabbit anti-profilin (Alexis Biochemicals).

Human tissues

Frozen blocks of human cortical tissue from Brodmann area 9 were obtained for Western blotting from the Harvard Brain Tissue Resource Center and Vanderbilt University Medical Center in accordance with institutional guidelines (NIH, Office of Human Subjects Research). Specimens were matched for postmortem interval (PMI) and divided into groups based on supplied information: (a) controls ($n=3$, age $=65\pm16$ years, PMI $=10\pm2$ h); (b) presymptomatic HD gene carriers ($n=3$, age $=46\pm31$ years, PMI $=13\pm6$ h); (c) grade 1 HD patients ($n=3$, age $=60\pm10$ years, PMI $=13\pm7$ h); (d), grade 4 HD patients ($n=3$, age $=67\pm6$ years, PMI $=16\pm7$ h). Neuropathological classification was based on previously established guidelines (Vonsattel et al., 1985). The mutant alleles in the HD patients ranged from 45 to 50 CAGs.

Cell culture

The cell model used for these experiments, a stably transfected, inducible PC12 cell line expressing exon 1 of the HD gene with either 23 or 74 CAGs under the control of a tetracycline promoter, has been previously described (PC12-23Q and PC12-74Q, (Wyttenbach et al., 2001)). The cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 10% horse serum (HS), 5% Tet-free fetal bovine serum (FBS), and 100 μg/ml G418 at 37 °C and 10% CO_2. Induction of htt expression was done by incubating the cells in media containing 1 μg/ml of doxycycline for the specified periods of time. For differentiation, the cells were incubated with 100 ng/ml of nerve growth factor (NGF) in media containing 1% HS and no FBS.

F actin sedimentation

The htt-inducible PC12 cells were incubated in media containing 1 μg/ml doxycycline for 48 h. For the specified experiments, cells were transfected with empty vector (mock) or with profilin-1 cDNA using Fugene HD as per the manufacturer's instructions (Roche). Following induction cells were scraped, washed in phosphate-buffered saline (PBS), and then lysed in 0.75 ml of actin lysis buffer (50 mM NaCl, 5 mM MgCl2, 1 mM ATP, 5% glycerol, 5 mM EGTA, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.9). The F actin and G actin pools were separated by ultracentrifugation at 100,000 ×g at 30 °C. The supernatant was diluted 1:2 with Laemmli buffer, and the pellet was resuspended in cold distilled H_2O with 1 μM cytochalasin D and sonicated for 10 s. The pellet fraction was kept on ice for 45 min and then diluted 1:4 with Laemmli buffer. Both fractions were then boiled for 5 min and centrifuged at 14,000 ×g for 10 min at 4 °C to remove remaining connective tissue. The same relative amounts of supernatant and pellet fractions (2:1) were loaded on 10% polyacrylamide gels and analyzed by Western blot using an anti-actin antibody (AC-40; Sigma-Aldrich). The F/G actin ratio was determined by scanning densitometry. The values are presented as the means ± standard error. Statistical comparisons were done using Student's *t*-test (p values <0.05 were considered statistically significant).

Cell death

The htt-inducible PC12 cells were seeded in 35 mm dishes at 4×10^5 cells/well. On day 1, the cells were transfected with empty vector (mock) or with profilin-1 cDNA using Fugene HD as per the manufacturer's instructions (Roche). The following day, cells were induced with 1 μg/ml doxycycline. 72 h following induction, the cells were trypsinized and centrifuged at 800 ×g for 5 min at room temperature. The cell pellets were washed and resuspended in 400 μl PBS. Propidium iodide (PI) was added to the cell suspension at 1 μg/ml. A total of 50,000 cells were collected and analyzed by flow cytometry (Becton Dickinson FACS Calibur). PI positive cells were detected using a 530 nm excitation and 617 nm emission filter. Cell populations were gated on size (forward scatter) and granularity (side scatter) to exclude debris and cell clumps. An uninduced, unstained negative control sample and a positive control sample stained with PI were used to establish reference regions. Cell death was calculated as the percent cells that were PI positive and reported as the mean ± standard error of three independent experiments. Statistical comparisons were performed using Student's *t*-test (p values <0.05 were considered statistically significant).

Western blotting

For human specimens, pieces of frozen cortical tissue were immersed in liquid nitrogen and pulverized with a BioPulverizer™ (Biospec Products, Bartlesville, OK). Powdered tissue was homogenized in a buffer consisting of 25 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% SDS, and protease inhibitors (Complete Mini, Roche) at 1:10 w/v. The samples were sonicated on ice for 10 s three times and then centrifuged at 14,000 ×g for 10 min at 4 °C to remove any particulate matter. For cells, at specified time points, the samples were washed with PBS, scraped, collected, and lysed in ice-cold RIPA buffer (50 mM Tris–HCl, 1% NP40, 0.5% Na-DOC, 150 mM NaCl, 0.1% SDS, and 2 mM EDTA) containing protease inhibitors. The cell lysate was sonicated on ice and incubated for 20 min on ice. For *Drosophila*, flies were frozen on dry ice and the heads were collected on a frozen plate and transferred to an eppendorf tube with RIPA buffer (1:5 w/v). The heads were