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In vivo suppression of polyglutamine neurotoxicity by C-terminus of Hsp70-interacting protein (CHIP) supports an aggregation model of pathogenesis

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ABSTRACT

Perturbations in neuronal protein homeostasis likely contribute to disease pathogenesis in polyglutamine (polyQ) neurodegenerative disorders. Here we provide evidence that the co-chaperone and ubiquitin ligase, CHIP (C-terminus of Hsp70-interacting protein), is a central component to the homeostatic mechanisms countering toxic polyQ proteins in the brain. Genetic reduction or elimination of CHIP accelerates disease in transgenic mice expressing polyQ-expanded ataxin-3, the disease protein in Spinocerebellar Ataxia Type 3 (SCA3). In parallel, CHIP reduction markedly increases the level of ataxin-3 microaggregates, which partition in the soluble fraction of brain lysates yet are resistant to dissociation with denaturing detergent, and which precede the appearance of inclusions. The level of microaggregates in the CNS, but not of ataxin-3 monomer, correlates with disease severity. Additional cell-based studies suggest that either of two quality control ubiquitin ligases, CHIP or E4B, can reduce steady state levels of expanded, but not wild-type, ataxin-3. Our results support an aggregation model of polyQ disease pathogenesis in which ataxin-3 microaggregates are a neurotoxic species, and suggest that enhancing CHIP activity is a possible route to therapy for SCA3 and other polyQ diseases.

Introduction

The crowded intracellular environment presents obstacles to proper folding of proteins. Accordingly, cells contain a molecular chaperone network that facilitates protein folding and promotes degradation of misfolded proteins. Its importance in the nervous system is highlighted by various neurodegenerative disorders, in which protein conformational defects cause detrimental accumulation of misfolded or aggregated protein. Protein misfolding and aggregation may underlie neurodegeneration in diseases such as Parkinson's disease, Alzheimer's disease and the polyglutamine (polyQ) diseases (Taylor et al., 2002; Selkoe, 2004; Walsh and Selkoe, 2004). Although protein aggregation occurs in all of these diseases, how protein misfolding and aggregation damages neurons is unclear. In the polyQ diseases, moreover, it is unknown which protein species are neurotoxic: monomers, oligomers, or higher order aggregates.

Aggregation is a common byproduct of protein misfolding, suggesting that protein quality control (PQC) systems are important in countering diseases where aggregation occurs. Two PQC pathways implicated in disease are the molecular chaperone system and the ubiquitin–proteasome pathway (Bence et al., 2001; Hay et al., 2004; Marques et al., 2006). The cochaperone and ubiquitin ligase, CHIP, links these two systems. Through its N-terminus, CHIP binds heat shock proteins and modulates protein refolding (Ballinger et al., 1999; Connell et al., 2001). Through its C-terminus, CHIP ubiquitinates substrates and targets them for degradation (Connell et al., 2001; Meacham et al., 2001).

The dual roles of CHIP in chaperone- and ubiquitin-dependent pathways suggest that it might be important in neuronal PQC. Numerous studies support a role for CHIP in countering neurodegenerative diseases. For example, CHIP enhances the activity of parkin, a protein associated with recessive parkinsonism (Imai et al., 2002), and regulates the degradation of phosphorylated tau, a protein implicated in various neurodegenerative diseases (Dickey et al., 2006, 2007a). Although CHIP modulates the toxicity of several polyQ disease proteins (Jana et al., 2005; Miller et al., 2005; Al-Ramahi et al., 2006; Choi et al., 2007; Branco et al. 2008), the precise role of CHIP in polyQ diseases is difficult to delineate because it may regulate polyQ proteins differently depending on protein context (Al-Ramahi et al., 2006; Bulone et al., 2006; Dickey et al., 2007b; Branco et al. 2008).

Expansion of the polyQ domain in the ataxin-3 protein causes the polyQ disease, SCA3. While normal ataxin-3 can self-associate (Gales et al., 2005), polyQ expansion enhances its aggregation and promotes

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amyloid-like aggregate formation (Chow et al., 2004; Ellisdon et al., 2006). Here, we used transgenic mice expressing full-length expanded ataxin-3 (Goti et al., 2004) as a disease model of polyQ protein misfolding to: 1) define the role of CHIP in disease and 2) identify putative toxic protein species in SCA3. We find that CHIP regulates the level of expanded (pathogenic) ataxin-3 in neuronal cells, and the solubility of expanded ataxin-3 in the brain. Additionally, reduction in CHIP expression enhances expanded ataxin-3 toxicity *in vivo*. Since CHIP reduction exacerbates the disease phenotype and increases microaggregate levels in the brain, our results support an aggregation-based model of polyQ disease pathogenesis.

Methods

Mouse strains

Q71-B hemizygous mice, a mouse model of SCA3/MJD (Goti et al., 2004) were bred to *CHIP* haploinsufficient mice (Dai et al., 2003) to obtain Q71-B hemizygous transgenic/*CHIP* haploinsufficient mice ($Q^{+/-}C^{+/-}$). These F1 progeny were bred to *CHIP* haploinsufficient mice to obtain F2 generation Q71-B mice with zero, one or two *CHIP* alleles. Mice were genotyped by standard two-way PCR as previously described (Goti et al., 2004; Dai et al., 2003). Q71-B/*CHIP* mice were maintained on a mixed genetic background (C3H/HeJ/C57BL6×129SvEv/C57BL6). Survival analysis was performed by determining how many animals of each genotype died by 12 months of age, excluding animals that were euthanized for tissue collection at predetermined time points. This was plotted as the percentage of animals of that genotype still alive at a specific month of age.

Rotarod analysis

Mice were trained on an accelerating rotarod apparatus (Ugo Basile, Comerio, Italy) for 2 days prior to testing. Training consisted of acclimating animals to the rotarod for 2 trials per day with 10 min rest between trials. For testing, the apparatus was set to accelerate from 3 to 30 rpm over the course of 5 min. Animals performed 3 trials with at least 10 min rest between trials. Trials ended when animals fell off the rod, began to rotate passively (i.e. clinging to the rod as it rotated) or completed the trial.

Open field analysis

Animals were tested for spontaneous activity using the Open Field 16×16 Photobeam Activation System and Flex-Field software (San Diego Instruments, San Diego, CA). Naive animals were placed into a 41 cm×41 cm clear plastic box inside a 16×16 array of infrared beams, and movement was calculated as the number and sequence of beam breaks over the course of 30 min. Animals were tested under dark conditions during their dark cycle (between 18:30 and 22:30 h).

Statistical analysis

Statistical analyses were performed using Microsoft Excel with the Data Analysis ToolPak. Behavioral data were grouped by age and genotype, then tested for significance with a two-way ANOVA for independent samples. When main effects were significant at $p<0.05$, they were also tested by one-way ANOVA for independent samples, then significant differences were further tested post-hoc using Tukey's Honestly Significant Difference (HSD, calculated online at http://web.mst.edu/~psyworld/virtualstat/tukeys/tukeycalc.html).

Echocardiography

Echocardiography was performed in sedated mice as previously described (Weiss et al., 2006). Mice were sedated with midazolam, and a 15 MHz linear array probe was applied horizontally to the chest. The imaging probe was coupled to a Sonos 5500 imager (Philips Medical Systems, Bothell, WA), generating ~180–200 two-dimensional frames per second in both short- and long-axis LV planes. Images were acquired and analyzed in blinded fashion with custom-designed software (Freeland Medical Systems, Louisville, CO). Endocardial and epicardial borders were traced in the short-axis plane at end-diastole and end-systole. The lengths from left ventricular outflow tract to endocardial apex and epicardial apex, respectively, were measured at end-diastole and end-systole. The bi-plane area-length method (Hill et al., 2000) was used to calculate end-diastolic and end-systolic left ventricular volumes and ejection fraction.

Immunohistochemistry and immunofluorescence

Mice were perfused transcardially with cold sterile PBS followed by 4% paraformaldehyde in PBS. Brains were fixed overnight in 4% paraformaldehyde, rinsed in PBS overnight, then cryoprotected in 30% sucrose for at least 18 h at 4 °C. Brains were cut serially into 30 μm floating sections on a sledge microtome (Leitz) and stored at −20 °C in cryoprotectant (5 mM sodium phosphate pH 7.4, 75 mM sodium chloride, 30% w/v sucrose, 30% v/v ethylene glycol) until use. For DAB immunohistochemistry with polyclonal antibodies, sections were blocked in H_2O_2 to quench endogenous peroxidase activity, then blocked in 5% normal goat serum and incubated in primary antibody (rabbit anti-MJD 1:1000 (Paulson et al., 1997) or rabbit anti-ubiquitin 1:500 (Dako USA, Carpinteria, CA)). Sections were rinsed and incubated in biotinylated goat-anti-rabbit 1:500 (Vector Laboratories, Burlingame, CA), then developed in 0.5 mg/ml DAB (Sigma, St. Louis, MO) with 9% H_2O_2, rinsed and mounted on gelatin-coated slides. For DAB immunohistochemistry with monoclonal GFAP-Cy3 antibody (Sigma), we used the Mouse-on-Mouse Peroxidase Kit (Vector Laboratories) according to the manufacturer's instructions. To quantify ubiquitin-positive inclusions, every 24th section of the brain was stained for ubiquitin and the total number of inclusions in sections of pontine nuclei was counted. For immunofluorescence, samples were fixed in 4% paraformaldehyde, rinsed in PBS, then incubated overnight with primary antibodies in 5% normal goat serum: anti-FLAG antibody for cell culture experiments (mouse anti-FLAG M5, Sigma, 1:500) or, for brain immunofluorescence, a combination of rabbit anti-ubiquitin (Dako, 1:500) and mouse anti-ataxin-3 (1H9, provided by Y. Trottier, 1:500). The next day, samples were rinsed and incubated in fluorescent secondary antibodies (goat anti-mouse Alexa 488 with or without goat anti-rabbit Alexa 568, Molecular Probes, Carlsbad, CA, 1:500) and nuclei were counterstained with DAPI (Sigma). Cells were coverslipped and sealed with Vectashield Hard-Set fluorescence mounting medium (Vector Laboratories).

Tissue collection and lysates

Mice were perfused transcardially with 20 ml cold sterile PBS plus protease inhibitor cocktail (PI) (Complete Mini tablets, Roche, Indianapolis, IN). Whole tissues were dissected, minced in RIPA (50 mM Tris–HCl pH 7.4, 150 mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) with PI, then homogenized in a Dounce homogenizer in RIPA plus PI lysis buffer. Lysates were diluted to 100 mg/ml (based on wet weight) in RIPA plus PI lysis buffer and centrifuged at 4000 rpm for 15 min at 4 °C. The pellet was rehomogenized in the same volume of RIPA plus PI. Lysates were stored at −80 °C until use.

Western blot analyses

Soluble fractions were sonicated twice for 10 s each, then spun at 15,000 ×g for 10 min at 4 °C to remove particulate debris (these pellets were not saved). Pellets from first 4000 rpm spin were vortexed and