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Research Article

Huntingtin interacting protein HYPK is a negative regulator of heat shock response and is downregulated in models of Huntington's Disease

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ABSTRACT

Huntingtin interacting protein HYPK (Huntingtin Yeast Partner K) is an intrinsically unstructured protein having chaperone-like activity and can suppress mutant huntingtin aggregates and toxicity in cell model of Huntington's Disease (HD). Heat shock response is an adaptive mechanism of cells characterized by upregulation of heat shock proteins by heat-induced activation of heat shock factor 1 (HSF1). The *trans*-activation ability of HSF1 is arrested upon restoration of proteostasis. We earlier identified HYPK as a heat-inducible protein and transcriptional target of HSF1. Here we show that HYPK can act as negative regulator of heat shock response by repressing transcriptional activity of HSF1. As part of its role as a repressor of heat shock response, HYPK can also inhibit HSF1-dependent *trans*-activation of its own promoter. HYPK is downregulated in cell and animal model of HD. We further show that transcriptional downregulation of HYPK in HD cell model is a consequence of reduced occupancy of HSF1 in *HYPK* promoter. Moreover, presence of mutant huntingtin inhibits effective induction of HYPK in response to heat shock. Taken together, our findings reveal that HYPK can suppress heat shock response via an autoregulatory loop and downregulation of HYPK in HD is caused by impaired transcriptional activity of HSF1 in presence of mutant huntingtin.

1. Introduction

Huntingtin Yeast two-hybrid Protein K (HYPK) was first identified from yeast-two-hybrid assay as an interacting partner of Huntingtin, the protein mutated in Huntington's Disease (HD) [1]. HD is a late-onset neurodegenerative disorder caused by mutation in exon 1 of *huntingtin* gene resulting in formation of mutant Huntingtin (HTT) protein having expanded polyglutamine stretch at its N-terminal [2]. HYPK is an intrinsically unstructured protein with a pre-molten globule-like conformation [3]. It has been shown that HYPK has *in vitro* and *in vivo* chaperone-like activity and has the ability to reduce aggregates of mutant HTT and subsequent toxicity in HD cell model [4,5]. A recent study reveals that a conserved nascent peptide-binding domain present in C-terminal of HYPK is required for its chaperone activity [6].

Over the years, HYPK has been shown to regulate diverse cellular events. The first evidence of involvement of HYPK beyond HD biology came in 2005 when HYPK was found to be associated with mammalian ribosome-associated complex [7]. In 2010, Arnesen et al., reported presence of HYPK in polysome fractions [8]. The same study showed that HYPK interacts with N(alpha)-terminal-acetyltransferase (NatA) complex and participates in co-translational N-terminal acetylation of different NatA substrates [8]. HYPK interacts with 37 proteins and regulates many biological processes including apoptosis, cell cycle regulation, response to unfolded proteins *etc.* [5]. The fact that knockdown of HYPK resulted in apoptosis and cell cycle arrest further suggests that HYPK is essential for survival and normal functioning of cells [5,8].

Heat shock proteins (HSPs) have long been identified as suppressors of cellular toxicity associated with different proteinopathies including HD [9,10]. HSPs can act as molecular chaperones and exert their cytoprotective activity either by reverting the misfolded proteins back into their native form or by facilitating the degradation and clearance of aggregates of misfolded proteins by autophagy and/or ubiquitin-proteasome pathway [11–13]. The precise role of intracellular aggregates in HD pathogenesis is still

Abbreviations: AcH4, acetylated histone H4; ChIP, chromatin immunoprecipitation; HD, Huntington's Disease; HTT, Huntingtin; HSE, heat shock element; HSF1, heat shock factor 1; HSPs, heat shock proteins; HSR, heat shock response; HYPK, Huntingtin Yeast Partner K; qRT-PCR, quantitative real-time PCR.

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controversial [14]; however, ample evidence suggests that suppression of these aggregates can protect cells from mutant HTT-induced toxicity [15,16]. Majority of the HSPs are induced by heat shock and transcriptionally regulated by Heat shock factor 1 (HSF1) [17]. Heat shock-dependent activation of HSF1 is a well-orchestrated multistep process and results in robust yet transient synthesis of multiple HSPs and other cytoprotective proteins [18,19]. Upon withdrawal of stress or restoration of proteostasis, transcriptional activity of HSF1 is attenuated and it goes back to inactive state. The mechanism of attenuation of HSF1 activity is still poorly understood. However, accumulation of some of the newly synthesized HSPs has been shown to inhibit *trans*-activation ability of HSF1 [20,21].

Earlier we reported that HYPK expression is induced by thermal stress and regulated by HSF1 in human and mouse cells [22]. In the present study, we demonstrated that HTT-interacting protein HYPK can act as a suppressor of heat shock response by repressing the activity of transcriptionally competent HSF1. We also showed that downregulation of HYPK in HD cell model is caused by reduced occupancy of HSF1 in *HYPK* promoter. In addition to reduced endogenous expression of HYPK, HD cell model also exhibited impaired heat shock-driven induction of *HYPK*, revealing the inhibitory effect of mutant HTT on heat shock response.

2. Materials and methods

2.1. Antibodies and chemicals

Anti-HSF1, anti-Hsp27, anti-Hsc70, anti-Hsp70 and anti-acetylated histone H4 antibodies were obtained from Abcam. Anti-Hsp40 and anti-Hsp60 antibodies were purchased from Cell Signaling Technology. Anti-Hsp90 antibody was purchased from BD Biosciences and anti-RNA polymerase II antibody was obtained from Imgenex. Anti-HYPK and anti-β-actin antibodies were procured from Sigma. The anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Bangalore Genei (India). Immobilon-P Transfer membrane was purchased from Milipore; Taq polymerase was purchased from Bioline and restriction enzymes used for cloning were obtained from New England Biolabs (NEB). Protease inhibitor mixture was procured from Roche Applied Science. TRIzol reagent, Lipofectamine 2000 and antibiotic G418 were purchased from Invitrogen. Other molecular biology grade fine chemicals used in this study were procured locally.

2.2. Cell culture and treatments

HeLa and Neuro2A (abbreviated as N2A) cells were procured from National Cell Science Centre, Pune, India and grown in Minimal Essential Medium (Himedia, India) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest), antibiotics penicillin/streptomycin (PS) 1% (v/v) and 400 μg/ml G418 at 37 °C in 5% CO_2 atmosphere under humified conditions. ST*Hdh*^{Q7}/*Hdh*^{Q7} and ST*Hdh*^{Q111}/*Hdh*^{Q111} cells were obtained from Dr. Marcy E MacDonald, Massachusetts General Hospital and grown in Dulbecco's Modified Eagle Medium (Himedia, India) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest), antibiotics penicillin/streptomycin (PS) 1% (v/v) and 400 μg/ml G418 at 33 °C in 5% CO_2 atmosphere under humified conditions. To induce heat shock response cells were subjected to heat shock at 42 °C for 60 min. For recovery of cells exposed to heat shock, cells were grown at 37 °C for indicated time periods. Transfection of cells was performed using Lipofectamine 2000.

2.3. R6/2 mice

Ovarian transplanted hemizygote females carrying exon 1 of *huntingtin* gene with about 150 CAG repeats [strain name B6CBA-Tg (Hdexon1)62Gpb/3J] were purchased from Jackson Laboratory and crossed with B6CBAF1/J males. The transgenic strain was maintained by crossing carrier males with CBA females. The genotyping was carried out using PCR as described previously by Mangiarini et al. [23]. All animal experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee of National Brain Research Centre, Manesar. Animals had free access to pelleted diet and water *ad libitum*. All efforts were made to minimize animal suffering. The HD transgenic mice along with their age-matched controls were anesthetized and then perfused with phosphate-buffered saline (PBS) containing 4% PFA in PBS, brain samples were collected and processed for paraffin embedding followed by cryosectioning with 20 μm thickness.

2.4. Construction of plasmids

Human *HSF1* gene cloned in pcDNA3.1 vector was kindly gifted by Dr. Richard Voellmy (HSF Pharmaceuticals, Switzerland). Cloning of human *HYPK* gene in GFP-tagged vector (HYPK-GFP) was described earlier [4]. Cloning of *huntingtin* exon 1 with 16 (wild-type) and 83 (mutant) CAG repeats in DsReD-C1 vector (BD Biosciences, USA) was described previously [4,24]. Cloning of human *HYPK* and *hsp70* promoter encompassing HSF1-binding sites (designated as HYPK_ups and Hsp70_ups respectively) were previously described [22].

For functional validation of the putative HSE present in −208 to −194 region of mouse *HYPK* promoter, −300 to +80 region of mouse *HYPK* promoter (designated as mHYPK_ups) was cloned in pGL3 basic vector (Promega). Similarly, promoter region of mouse *hsp70* (*HSPA1B*) gene (−196 to +114 region) encompassing active HSE at −110 to −96 region was also cloned in pGL3 basic vector and designated as mHsp70_ups. The primer sequences used for cloning are given in Supplementary Table S1.

2.5. Luciferase assay

The method used for luciferase assay was described earlier [22,25]. Briefly, cells were transfected with 600 ng of different reporter constructs and 400 ng of different pcDNA3.1 constructs (empty pcDNA 3.1 vector and HSF1-pcDNA). Twenty four hours after transfection, cell extract was prepared in presence or absence of heat shock treatment depending on experimental designing and luciferase reporter assay was done using the luciferase reporter assay system (Promega) according to manufacturer's protocol and detected by a Sirius Luminometer (Berthold Detection Systems). The experiments were performed in triplicate.

2.6. RNA preparation

Total RNA was prepared from cultured cells using TRIzol reagent according to manufacturer's protocol. The method of RNA extraction from paraffin embedded tissue samples of R6/2 mice and wild-type mice were based on the protocol described by Stanta et al. [26] and Korbler et al. [27] and also mentioned earlier [28].

2.7. Quantitative real time PCR (qRT-PCR)

One microgram RNA was subjected to DNase treatment (Sigma) followed by cDNA preparation using random hexamer primer, dNTPs and MuLv-Reverse transcriptase (Fermentas). Quantitative real time PCR (qRT-PCR) was carried out using Sybr green 2X Universal PCR Master Mix (Applied Biosystems) in 7500 Real time PCR system (Applied Biosystems). For each gene non-template control was used at the same condition to ascertain the baseline and threshold value for the analysis. The absolute quantification given by the software was in terms of C_t values. The relative quantification (fold change) of a target gene in a sample compared to parental cell is expressed in terms of $2^{-\Delta\Delta Ct}$ values after normalization with respect to internal control (*β-actin* gene). The gene (mRNA)-specific primers were designed using Primer Express software. The list of primer sequences is given in Supplementary Table S2.

2.8. Western blot

Preparation of protein extract from mice brain parts was carried out as detailed in [29]. Western blot was carried out following the method described earlier [22]. Expression of β-actin was considered as loading control. Each experiment was repeated three times. Integrated optical density (IOD) of each band was calculated by Image Master VDS software (Amersham Bioscience).

2.9. Chromatin immunoprecipitation

Method used for chromatin immunoprecipitation (ChIP) assay was described earlier [22]. Cells were grown at 33 °C/37 °C (no heat shock) or subjected to standard heat shock treatment according to experimental need. Immunoprecipitation was done using anti-HSF1, anti-RNA polymerase II and anti-acetylated histone H4 (AcH4) antibodies. Finally the eluted DNA was amplified by semi-quantitative and quantitative RT-PCR using primers complementary to mouse *HYPK* and *hsp70* promoter bearing putative or functionally active HSE. A fragment of the mouse genome having no HSE was also amplified using the immunoprecipitated DNA and this non-specific sequence (NS seq) was used as negative control. Primer sequences used in ChIP assay are given in Supplementary Table S3. Quantification was done by normalizing the immunoprecipitated DNA to the input DNA in each sample and fold enrichment was calculated by considering the normalized immunoprecipitated DNA in unstressed cells (control) as 1.