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Cellular toxicity of yeast prion protein Rnq1 can be modulated by N-terminal wild type huntingtin

Ratnika Sethi, Vishal Patel, Aliabbas A. Saleh, Ipsita Roy*

Department of Biotechnology, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar, Punjab 160062, India

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ABSTRACT

Aggregation of the N-terminal human mutant huntingtin and the consequent toxicity in the yeast model of Huntington's disease (HD) requires the presence of Rnq1 protein (Rnq1p) in its prion conformation [RNQ1$^+$]. The understanding of interaction of wild-type huntingtin (wt-Htt) with the amyloidogenic prion has some gaps. In this work, we show that N-terminal fragment of wt-Htt (N-wt-Htt) ameliorated the toxic effect of [RNQ1$^+$] depending on expression levels of both proteins. When the expression of N-wt-Htt was high, it increased the expression and delayed the aggregation of [RNQ1$^+$]. As the expression of N-wt-Htt was reduced, it formed high molecular weight aggregates along with the prion. Even when sequestered by [RNQ1$^+$], the beneficial effect of N-wt-Htt on expression of Rnq1p and on cell survival was evident. Huntingtin protein ameliorated toxicity due to the prion protein [RNQ1$^+$] in yeast cells in a dose-dependent manner, resulting in increase in cell survival, hinting at its probable role as a component of the proteostasis network of the cell. Taking into account the earlier reports of the beneficial effect of expression of N-wt-Htt on the aggregation of mutant huntingtin, the function of wild-type huntingtin as an inhibitor of protein aggregation in the cell needs to be explored.

1. Introduction

Huntington's disease (HD) is a dominantly inherited disorder and is caused due to expansion of the polyglutamine stretch in the N-terminus of the multi-domain protein called huntingtin [51]. The formation of aggregates has been shown to correlate well with the length of the polyglutamine repeat in the protein [26,39]. The sequestration of various cellular factors, including the wild type huntingtin ('loss of function' hypothesis), by the growing clump of mutant huntingtin, leads to disruption of various cellular pathways and finally cell death. However, the role of inclusions themselves in disease progression remains ambiguous. While a majority of reports seem to link the extent of aggregation directly with toxicity in cells, a few equally convincing studies suggest an initial cytoprotective role for the inclusions [1,33]. The budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryote, is a useful tool to study fundamental cellular and molecular processes and has been used extensively as a model organism to study many protein misfolding disorders including HD. Both genetic and physical interactions involving huntingtin and basic cellular mechanisms such as transcription, mitochondrial function, vesicular trafficking, apoptosis, etc. can be reproduced faithfully in this model [6,26,31,32,50].

Although toxicity is associated with formation of inclusions in mammalian cells, the expression of heterologous mutant huntingtin, with an expanded polyglutamine tract, in yeast is not sufficient to reproduce the toxicity associated with HD even though visible aggregates are formed. The presence of prions, especially Rnq1p, in the amyloid conformation [RNQ1$^+$], seems to be a necessary condition for aggregation to cause toxicity in yeast cells [31]. Prions are infectious protein isoforms in yeast that are transmitted via non-Mendelian mode of inheritance. Propagation occurs by 'seeding' of the prion by the yeast protein remodelling factor, Hsp104, which fragments the amyloid conformation, releases nuclei which segregate into the budding daughter cells and continue conversion of benign conformations into prions [14,40,43,44,52]. This non-Mendelian mode of inheritance is similar to the 'protein-only' replication model proposed for mammalian prions and many other proteins associated with many neurodegenerative disorders [12]. Apart from Rnq1p, a number of other prion-forming proteins have been reported in *S. cerevisiae*, including Ure2, Sup35, Swi, etc. Some have defined functions in the cell in the non-prion form; Rnq1p induces the formation of [PSI$^+$], the prion conformation of Sup35p [8]. Like most other yeast

* Corresponding author.
E-mail address: ipsita@niper.ac.in (I. Roy).

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prionogenic proteins, Rnq1p has an asparagine/glutamine-rich domain and folds into the amyloid conformation [45]. As the mechanism of 'replication' of prions is similar in mammalian and yeast cells, the latter provides a facile platform to study the modulators of prion propagation in the cell. In the absence of Rnq1p, the mutant huntingtin protein does not produce cytotoxicity in yeast which is characteristic of HD [31]. The interaction of Rnq1p with mutant huntingtin has been investigated rather extensively [11,24,30,31,46]. However, the functioning of the wild type protein in the cell is not well understood. Huntingtin knockout results in embryonic lethality in mice [35,56], the same is not observed in zebrafish [28] or Drosophila [57]. Instead, developmental defects are observed. The protein has been implicated in cell survival by providing resistance against pro-apoptotic signals [29,38]. The similarity of the C-terminal domain of the protein with a yeast autophagy scaffold protein has led to the suggestion that huntingtin could be a member of the autophagy pathway in the cell [36]. It is likely that huntingtin exerts pleiotropic effects in the cell by interacting with various partners via its different domains. The modulation of structures of polyglutamine-rich proteins like Rnq1p or mutant huntingtin and attenuation of the resultant cytotoxicity by wild-type huntingtin [5] may be one of them and needs to be explored further. The interaction, if any, of Rnq1p with the wild type huntingtin, which differs from the mutant protein only in the number of glutamine residues at the N-terminal domain, has not been reported. There are differing accounts of interaction between wild type and mutant huntingtin in the literature. Attenuation of toxicity of mutant huntingtin was observed in the presence of the wild-type counterpart in cell models [20] as well as a transgenic mouse HD model [27]. On the other hand, mutant huntingtin formed detergent-insoluble amyloid-type coaggregates with wild-type huntingtin [4]. Proteins containing polyglutamine-rich tracts interact with each other by the formation of polar zippers [21,37]. This has been documented in case mutant huntingtin acting as a modulator of transcription factors by sequestering them. A recent report has shown that the expression level of each protein is a critical determinant of its solubility and decides the toxicity in the cell harbouring them [42]. Since both Rnq1p and wild type huntingtin contain the polyQ domain, we have studied the effect of the presence of wild type huntingtin on the aggregation of Rnq1p and vice versa and the consequences on yeast cell viability.

2. Materials and methods

S. cerevisiae BY4742 parental and Δ*Hsp104* strains were products of Open Biosystems and were obtained from SAF Labs Pvt. Ltd., Mumbai, India. Luria bertani broth, ampicillin, phenylmethanesulphonyl fluoride (PMSF), mouse anti-polyQ antibody, goat anti-mouse FITC conjugated antibody and anti-goat FITC conjugated antibody were purchased from Sigma–Aldrich, Bangalore, India. Goat anti-Rnq1 antibody was purchased from Santa Cruz Biotechnology, USA. Succinic acid and amino acids for amino acid dropout mixtures were purchased from SRL Pvt. Ltd., Mumbai, India. Yeast nitrogen base (without amino acids) was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Goat anti-mouse horseradish peroxidase (HRP) conjugated monoclonal antibody and tetramethyl benzidine/hydrogen peroxide substrate were obtained from Bangalore Genei, Bangalore, India. One step SYBR® Ex Taq™ qRT-PCR (Perfect Real Time) kit was a product of Takara Bio Inc., Japan. All other reagents and chemicals used were of analytical grade or higher.

2.1. Plasmids

pYES2 contains the 2μ origin of replication, *URA3* selection marker and induces the expression of the heterologous protein via the *GAL1* promoter [31]. Inserts cloned into this plasmid were expressed in high copy number in yeast cells. pRS315 is a mitotically stable yeast replicating plasmid carrying the yeast centromere sequence (*CEN*) and autonomously replicating sequence (*ARS*) [48]. It contains the *LEU2* selection marker and regulates the expression of the heterologous protein via the *CUP1* promoter [12]. Inserts cloned into this plasmid were expressed in low copy number in yeast cells. The vectors pYES2-*25Q-Htt-EGFP* [31], pRS315-*Rnq1-mRFP* [11], pRS315-*25Q-Htt-mRFP* [42] and pRS315-*mRFP* [44] have been described earlier. For construction of pYES2-*Rnq1-EGFP*, pRS315-*Rnq1-mRFP* was subjected to PCR amplification of *Rnq1* with a forward primer containing *Kpn*I restriction site and a reverse primer containing *Bam*HI restriction site. The vector pYES2-*25Q-Htt-EGFP* was digested with the same enzymes (Fig. 1A). The amplified *Rnq1* insert was ligated with the double digested plasmid. The integrity of the recombinant plasmid was confirmed by restriction digestion (Fig. 1B). The empty vector pYES2-*EGFP* was constructed by digesting pYES2-*25Q-Htt-EGFP* with *Kpn*I and *Bam*HI to delete *25Q-Htt*. The gel-purified, linearized vector was ligated to a stuffer oligonucleotide sequence containing flanking *Kpn*I and *Bam*HI restriction sites (Fig. 1C). The deletion of *25Q-Htt* was confirmed by agarose gel electrophoresis (Fig. 1D).

2.2. Expression of Rnq1p and 25Q-Htt in yeast cells

S. cerevisiae BY4742 cells (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; [RNQ1^{+}]) (parental and deletion strains) were transformed with plasmids expressing Rnq1p (pYES2-*Rnq1-EGFP* or pRS315-*Rnq1-mRFP*), 25Q-Htt (pYES2-*25Q-Htt-EGFP* or pRS315-*25Q-Htt-mRFP*), both or with the respective empty vectors, as indicated, following a standard protocol [17]. Transformed cells were grown in SC-URA, SC-LEU or SC-URA-LEU (for cotransformed cells) media containing 2% dextrose and grown at 30 °C. Expression of the respective proteins was induced with 50 or 500 μM $CuSO_4$ (for low copy number pRS315 vector), 2% galactose (for high copy number pYES2 vector) or both in appropriate selection media for the indicated time intervals and monitored by confocal microscopy. Cells were lysed by the glass beads method [15]. Protein estimation was carried out in the resulting supernatant by dye binding method [3].

2.3. Native PAGE analysis

Samples were analysed by native polyacrylamide gel electrophoresis. Gels were scanned on an image scanner (Typhoon Trio, GE Healthcare, Sweden) in the fluorescence mode for detection of GFP or RFP signal.

2.4. Semi-denaturing detergent-agarose gel electrophoresis (SDD AGE)

Aggregated proteins were visualized using 1.5% agarose gel containing 0.1% SDS [19]. Samples were incubated with 1X TAE (Tris acetate-EDTA) containing 0.1% SDS and tracking dye for 5 min without heating prior to loading onto the gel. Post-electrophoresis, samples were transferred electrophoretically onto a nitrocellulose membrane (0.45 μm) using a semi dry blotter (TE 70 PWR, GE Healthcare). Bound protein was visualized using polyQ antibody (1:500) as the primary antibody and FITC-conjugated goat anti-mouse antibody (1:2500) as the secondary antibody. Bands were detected using an image scanner (Typhoon Trio) operated in the fluorescence mode.