Journal of Biotechnology 239 (2016) 1–8

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Cell-free analysis of polyQ-dependent protein aggregation and its inhibition by chaperone proteins

Kodai Machida[a,b,*], Tomoaki Shigeta[a], Ayano Kobayashi[a], Ai Masumoto[a], Yuna Hidaka[a], Hiroaki Imataka[a,b,*]

[a] Department of Applied Chemistry, Graduate School of Engineering, University of Hyogo, Himeji 671-2201, Japan
[b] RIKEN Center for Life Science Technologies, Yokohama 230-0045, Japan

ARTICLE INFO

Article history:
Received 20 August 2016
Received in revised form
23 September 2016
Accepted 30 September 2016
Available online 1 October 2016

Keywords:
Cell-free
In vitro translation
Polyglutamine
Aggregation
Chaperone
CCT

ABSTRACT

Protein misfolding and aggregation is one of the major causes of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease. So far protein aggregation related to these diseases has been studied using animals, cultured cells or purified proteins. In this study, we show that a newly synthesized polyglutamine protein implicated in Huntington's disease forms large aggregates in HeLa cells, and successfully recapitulate the process of this aggregation using a translation-based system derived from HeLa cell extracts. When the cell-free translation system was pre-incubated with recombinant human cytosolic chaperonin CCT, or the Hsc70 chaperone system (Hsc70s: Hsc70, Hsp40, and Hsp110), aggregate formation was inhibited in a dose-dependent manner. In contrast, when these chaperone proteins were added in a post-translational manner, aggregation was not prevented. These data led us to suggest that chaperonin CCT and Hsc70s interact with nascent polyglutamine proteins co-translationally or immediately after their synthesis in a fashion that prevents intra- and intermolecular interactions of aggregation-prone polyglutamine proteins. We conclude that the *in vitro* approach described here can be usefully employed to analyze the mechanisms that provoke polyglutamine-driven protein aggregation and to screen for molecules to prevent it.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Cell-free (*in vitro*) translation systems have proven to be remarkably powerful tools in various biochemical studies. Notable examples include the milestone study to identify the genetic code (Nirenberg and Matthaei, 1961) and a cell-free system to demonstrate *de novo* poliovirus replication (Barton et al., 1995; Molla et al., 1991). Cell-free translation systems are now indispensable for understanding the mechanisms of not only translation, but also of co-translational protein folding (Niwa et al., 2012). Cell-free translation systems have tangible advantages over protein expression systems using living cells. The absence of a cell membrane facilitates direct supplementation with various substances, allowing researchers to easily modify and observe translation and translation-related processes. Moreover, there are no worries about cell viability and proliferation. Thus, it is conceivable that studies using an *in vitro* translation system may be expanded to include analyses of protein modification and aggregation that can ultimately provoke cellular dysfunction and death.

The accumulation of protein aggregates is a hallmark of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease. Huntington's disease is caused by mutant Huntingtin protein, encoded by a transcript generated from the short arm of chromosome 4, position 16.3 (Collaborative and Group, 1993). Although the normal function of Huntingtin remains obscure, the lengths of the polyglutamine (polyQ) repeats expressed at the N-terminus of Huntingtin directly correlate with its aggregation-prone nature (Scherzinger et al., 1997) (Penney et al., 1997). A genome-wide RNA interference screen in *C. elegans* identified 186 genes that were associated with polyQ-dependent aggregation (Nollen et al., 2004) together with genes that encode the mammalian homologues of chaperone pro-

Abbreviations: NAC, nascent polypeptide-associated complex; RAC, ribosome-associated complex; PFD, prefoldin; CCT, chaperonin containing TCP-1; Hsc70, heat shock cognate 71 kDa protein; Hsc70s, Hsc70 chaperone system.

* Corresponding authors at: Department of Applied Chemistry, Graduate School of Engineering, University of Hyogo, Himeji 671-2201, Japan.
E-mail addresses: machida@eng.u-hyogo.ac.jp (K. Machida), imataka@eng.u-hyogo.ac.jp (H. Imataka).

http://dx.doi.org/10.1016/j.jbiotec.2016.09.031
0168-1656/© 2016 Elsevier B.V. All rights reserved.

teins Hsp40, Hsp70 and subunits of chaperonin CCT. Indeed, several studies have shown that the molecular chaperones including small Hsps (Krobitsch and Lindquist, 2000; Wyttenbach et al., 2002), Hsp40 and Hsp70 (Krobitsch and Lindquist, 2000; Muchowski et al., 2000; Wacker et al., 2004; Warrick et al., 1999), prefoldin (PFD) (Tashiro et al., 2013), chaperonin CCT (Behrends et al., 2006; Kitamura et al., 2006; Nadler-Holly et al., 2012; Tam et al., 2006, 2009) and Hsp104 (Kimura et al., 2004; Krobitsch and Lindquist, 2000) can remodel the aggregation status of the polyQ protein and moderate cytotoxicity. These studies have been carried out using animals, cultured cells or purified proteins. Here we propose that the mechanism of polyQ-dependent aggregation and its prevention by chaperone proteins in the context of protein synthesis could be studied more efficiently using an *in vitro* translation system.

We previously developed a HeLa cell extract-based cell-free translation system (Mikami et al., 2008, 2006), and used this to successfully analyze the replicative mechanisms of encephalomyocarditis virus (Kobayashi et al., 2011, 2012; Machida et al., 2014) and the functions of human chaperonin CCT (Machida et al., 2012; Ozdemir et al., 2016).

In this study, we now investigate the effects of the major human chaperones (RAC: Hsp70L1/Hsp70A1A/MPP11, NAC: NACα/NACβ, the Hsc70 system (Hsc70s): Hsc70/Hsp40/Hsp110, the Hsp90 system: p23/Hsp90, PFD, and CCT) on the formation of polyQ-derived aggregates using our HeLa cell extract-derived cell-free translation system. Our results demonstrate that the Hsc70 system and CCT suppress polyQ aggregation in a co-translational fashion.

2. Materials and methods

2.1. Plasmids

Huntingtin polyQ-GFP-HA: Plasmids encoding Huntingtin (HTT) polyQ-GFP sequences: p426 25Q GPD (ID:1181), p426 46Q GPD (ID:1182), p426 72Q GPD (ID:1183) and p426 103Q GPD (ID:1184) were purchased from Addgene (MA, USA). To facilitate detection of the gene products, an HA-tag sequence was fused to the C-terminus of each HTT polyQ-GFP sequence by polymerase chain reaction (PCR) followed by sub-cloning into the pCI-neo (Promega) and HCV IRES (Machida et al., 2014) plasmids to generate pCI-neo-25, 46, 72 and 96Q GFP-HA and HCV IRES 25, 46, 72 and 96Q GFP-HA. Note that the number of triplet CAA repeats (encoding glutamine: Q) in the plasmids derived from p426 103Q GPD was found to be 96, and not 103 by DNA sequencing.

Nascent chain associated complex (NAC: NACα and NACβ): Complementary DNAs (cDNAs) for the human NAC subunits (GenBank accession no. X80909 for NACα and X53281 for NACβ) were amplified from human testis RNA (Clontech) by reverse transcriptase (RT)-PCR. To facilitate purification of the NAC complex, a His-tag sequence was inserted at the C-terminus of the NACβ subunit. The cDNA for each subunit was sub-cloned into pUC-T7-EMCV-MCSter (Mikami et al., 2008) to generate pUC-T7-EMCV-NACα and NACβ −His.

Ribosome associated complex (RAC: MPP11, Hsp70L1 and Hsp70A1A): cDNAs for human MPP11 (acc. no. NM_014377) and Hsp70L1 (NM_016299) were amplified from human bone marrow RNA (Clontech) by RT-PCR. A cDNA for Hsp70A1A (NM_005345) was purchased from OriGene. Each cDNA with a GST sequence at the 5′ end was sub-cloned into the pFastBac vector (Life technologies) to generate pFastBac-GST-MPP11, −GST-Hsp70A1A and −GST-Hsp70L1.

Hsp40, Hsc70, Hsp110, Hsp90: cDNAs for human Hsp40 (acc. no. D49547), Hsc70 (AF352832), Hsp110 (NM_006644) and Hsp90 (NM_001017963) were amplified from human placenta poly(A) + RNA (Clontech) by RT-PCR. Again, cDNAs with a GST sequence at their 5′ termini were sub-cloned into the pFastBac vector (Life technologies) to generate pFastBac-GST-Hsp40, −GST-Hsc70, −GST-Hsp110 and −GST-Hsp90.

p23: a cDNA for human p23 (acc. no. AK298147) was amplified from human placenta poly(A) + RNA (Clontech) by RT-PCR, and was sub-cloned in pGEX-6P-1 (GE Healthcare) to generate pGEX-p23.

Prefoldin (PFD) 1-6: cDNAs for human PFD1 (acc. no. NM_002622), PFD2 (AF117237), PFD3 (Y17394), PFD4 (NM_002623), PFD5 (BC062671) and PFD6 (BC039033) were amplified from human placenta poly(A) + RNA (Clontech) by RT-PCR. To facilitate the purification of the PFD complex, a FLAG-tag sequence was appended to the N-terminus of the PFD1 subunit. These cDNAs were sub-cloned into pUC-T7-EMCV-MCSter (Mikami et al., 2008) to generate pUC-T7-EMCV-FLAG-PFD1, −PFD2,- PFD3,-PFD4, −PFD5 and −PFD6.

2.2. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai, Japan) supplemented with 10% fetal calf serum, 1% L-Alanyl-L-glutamine solution (Nacalai) and 1% Penicillin-Streptomycin Mixed solution (Nacalai) in 6-well cell culture plates (Falcon) in a 5% CO_2 humidified incubator. These cultures were transfected with pCI-neo-GFP-HA, pCI-neo-25Q-GFP-HA, pCI-neo-46Q-GFP-HA, pCI-neo-72Q-GFP-HA or pCI-neo-96Q-GFP-HA using Lipofectamine 2000 (Invitrogen) as follows: cells were grown to 50% confluence then washed with phosphate-buffered saline (PBS, 1 ml). A mixture of plasmid DNA (2 μg) and Lipofectamine 2000 (6 μl) prepared in OPTI-MEM solution (Gibco, 600 μl) was added to cells. After incubation for 3 h in a CO_2-gassed incubator, 2 ml of DMEM supplemented with 10% FCS was added to the cells, which were incubated for a further 14 h.

2.3. Fluorescence microscopy of polyQ-GFP-HA transfectants

After incubation for 17 h, transfected HeLa cells were observed with the CKX41 inverted microscope equipped with an epifluorescence system using the B-excitation mirror (Olympus, Japan) to detect the GFP fluorochrome expressed by polyQ GFP-HA aggregates.

2.4. Cell free expression of the polyQ-GFP-HA proteins

The polyQ-GFP-HA proteins were synthesized *in vitro* using the HeLa S3 cell extract-derived transcription/translation system (Mikami et al., 2008). Briefly, 7.5 μl of HeLa S3 extract (~8 mg/ml), 6.5 μl of Mixture-2 (97 mM Hepes-KOH pH7.5, 290 mM potassium acetate, 8.6 mM magnesium acetate and 12.7 mM DTT) and 1.2 μl of GADD34 (0.2 mg/ml) were mixed in a 1.5-ml microfuge tube. After incubation for 10 min at 32 °C, 1.8 μl of Mixture-1 (12 mM ATP, 8.2 mM GTP, 8.2 mM UTP, 8.2 mM CTP, 196 mM creatine phosphate, 1.2 mg/ml creatine kinase, 0.3 mM twenty-amino acids mixture, 5 mM spermidine, 0.1 mg/ml T7 RNA polymerase) and 1 μl of the HCV IRES 25,46,72 or 96Q GFP-HA plasmid (0.5 μg/μl) were added to the tube, and then incubated for the indicated time periods at 32 °C. Protein products were analyzed by the filter trap assay and sodium dodecyl sulfate (SDS)-PAGE followed by western blot.

2.5. Filter trap assay of polyQ-GFP-HA aggregates

For the biochemical detection of polyQ-GFP-HA aggregates formed *in vivo*, transfectants from a 6-well dish were harvested and lysed in 500 μl of PBS containing 1% Triton X-100 and 1% SDS. To apply an equal amount of 25,46,72, and 96Q-GFP-HA proteins from each sample to the filter trap assay, we monitored the expression levels for each polyQ-GFP-HA protein by western blot,

Download English Version:

https://daneshyari.com/en/article/6452244

Download Persian Version:

https://daneshyari.com/article/6452244

Daneshyari.com