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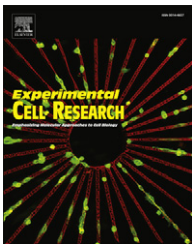

Research Article

Cotransin induces accumulation of a cytotoxic clusterin variant that cotranslationally rerouted to the cytosol

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ABSTRACT

Although clusterin (CLU) was originally identified as a secreted glycoprotein that plays cytoprotective role, several intracellular CLU variants have been recently identified in the diverse pathological conditions. The mechanistic basis of these variants is now believed to be alternative splicing and retrotranslocation. Here, we uncovered, an unglycosylated and signal sequence-unprocessed, CLU variant in the cytosol. This variant proved to be a product that cotranslationally rerouted to the cytosol during translocation. Cytosolic CLU was prone to aggregation at peri-nuclear region of cells and induced cell death. Signal sequence is shown to be an important determinant for cytosolic CLU generation and aggregation. These results provide not only a new mechanistic insight into the cytosolic CLU generation but also an idea for therapeutic mislocalization of CLU as a strategy for cancer treatment.

Introduction

In the mammalian cells, approximately one forth of proteins including extracelluar signaling molecules and protein receptors have destination to the ER. However, this could be perturbed in various disease conditions. Then, minor species of the proteins are often mislocalized to unexpected subcellular compartments. Although noncompartmentalized proteins are initially very small in amount and unimportant enough to ignore, their accumulation often leads to disease pathogeneses, when cellular degradation mechanisms fail to clean up those proteins. One such example is clusterin (CLU). Although majority of CLU is in secretory form (sCLU) that is now accepted as playing a role as a cytoprotective extracellular chaperone similar to small heat shock proteins [1,2], several intracellular CLU variants have been uncovered in various tissues and cells under various stimuli [3–5].

The mechanistic basis of intracellular CLU is now believed to be alternative splicing and retrotranslocation. Nuclear CLU (nCLU) (43–55 kDa) is mostly well-studied intracellular CLU isoform. This isoform is a spliced variant translated from the third ATG of multiple in-frame ATG codons of human CLU, encoding truncated CLU lacking hydrophobic signal sequence.

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It has been postulated that truncated CLU which failed to target the ER is rerouted into the nucleus due to nuclear localization signal (NLS) [3,6]. There are several evidences to suggest that the expression of this isoform is enhanced in apoptosis and by the treatment of transforming growth factor-β [3], ionizing radiation [4,7], tumor necrosis factor-α and anti-estrogen [5]. The enhanced expression and nuclear accumulation of nCLU appear to be a cell death signal rather than cytoprotective [7,8].

In addition to nCLU, several CLU variants that revealed heterogeneity in mass accumulate in the cells during ER stress. In an early study, multiple intracellular CLU variants have been detected in the cells treated with thapsigargin [9]. These variants were recently proved as a hypoglycosylated CLU retrotranslocated from the ER to the cytosol and suppressed paclitaxel-mediated apoptosis of human prostate cancer cells [10]. Moreover, an incompletely glycosylated 60-kDa variant of CLU has been found in the mitochondria of human cancer cells; this variant promotes oncogenic transformation and tumor progression by interfering pro-apoptotic activity of Bax [8,11]. Among the various possible reasons for these heterogeneities of intracellular CLUs, the explanation is to be an alteration of the post-translational modifications and intracellular trafficking of CLU [5]. This notion is further supported by our recent observation suggesting that N-glycan deficient CLU variant (~50 kDa) is retained in the ER and induces cytotoxicity [12].

CLU is cotranslationally translocated into the ER where it is folded and glycosylated. CLU biogenesis seems to be extremely complicated as the protein undergoes multiple modifications during its passage through the secretory pathway. In this context, CLU seems to be especially sensitive to ER stress, because the protein requires fine-tuned sequential engagements of targeting, processing, and folding machinery in the ER. However, our previous work suggested that translocation of most proteins synthesized in the ER is also regulated during ER stress [13] allows us to hypothesize that at least a small fraction of CLU would fail to be translocated into the ER lumen and potentially function in the cytosol.

To detect small amounts of CLU in the cytosol, we used compartment-restricted biotinylation assay that selectively biotinylates cytosolically exposed small species of mislocalized proteins [14]. Using this method, we could identify a new cytosolic CLU variant that is induced during ER stress and by the treatment of cyclodepsipeptide cotransin. This variant proved to be a cytotoxic product that cotranslationally rerouted to the cytosol during translocation rather than being generated by alternative splicing or retrotranslocation. Here, we provide a mechanistic insight into the generation of this variant and discuss about therapeutic potential of regulating CLU translocation for cancer treatments.

Methods

Plasmid constructions and antibodies

The 5′-phosphorylated synthetic oligos of HA epitope and PCR-amplified GFP were inserted into unique HindIII and EcoO109I sites of pcDNA5-FRT/TO (pcDNA5-FRT/TO-HA and -GFP, respectively). Wild-type human CLU and signal sequence-deficient mutant CLU derivative ($CLU^{\Delta SS}$) were cloned by RT-PCR from total RNA of HeLa cells and subcloned into pcDNA5-FRT/TO-HA or -GFP vector. Bovine prolactin (Prl), GFP and FLAG-BirA plasmids were provided by Dr. Hegde et al. (University of Cambridge, UK) [13,14]. Inserts from Prl and GFP were PCR-amplified and subcloned into pcDNA5-FRT/TO-HA and pcDNA5-FRT/TO vectors, respectively. Prl—CLU was engineered by PCR using synthetic oligos encoding Prl signal sequences. The 5′-phosphorylated synthetic oligos encoding BioTag epitope ("ALN-DIFEAQKIEWHA") was directly inserted into the unique sites of XhoI at pcDNA5-FRT/TO–CLU-HA. The following primary antibodies were used for this study: anti-HA (Covance, Princeton, NJ), anti-CLU (M18) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GRP78 (BD Bioscience, San Jose, CA), and anti-TRAPα and Sec61β, and PrP (kindly provided by Hegde).

Cell culture analysis

HeLa cells were purchased from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% CO_2 at 37 °C. For pulse-labeling experiments, cells transfected with BioTag/HA epitope-tagged CLU constructs were incubated with methionine free media for 15 min in the presence and absence of ER stress inducers or cotransin (CT), and pulse-labeled for additional 15 min with [^{35}S] methionine. Fractionation of cytosolic and glycoproteins was performed as described [13] with minor modifications. For confocal microscopy, HeLa cells were plated onto coverslips and transfected with constructs of wtCLU or $CLU^{\Delta SS}$ fused with GFP or GFP_{KDEL} for 8 h. Cells were incubated with an additional 12 h with or without CT and fixed, mounted according to the standard methods as described [12]. Microscopy was performed using a TCS-SP2 confocal laser-scanning microscope (Leica, Wetzlar, Germany).

Biotinylation assay in vivo

Cells were cotransfected with FLAG-BirA and HA/BioTag epitope-tagged wtCLU and $CLU^{\Delta SS}$ as 1:5 ratio of the total amount of DNA, and pulse-labeled for 15 min. Cells were fully solubilized with 1% SDS, 100 mM Tris–HCl, pH 8.0 and diluted 10-fold in IP buffer (100 mM NaCl, 50 mM HEPES, 1% Triton X-100). This was incubated with immobilized avidin–sepharose for 2 h. Beads were recovered with centrifugation and washed with IP buffer. Biotinylated CLU-HA was released from the beads with SDS sample buffer and analyzed directly from SDS-PAGE by autoradiography.

In vitro analysis

In vitro transcription, translation, protease digestion, and sedimentation assay were performed as described [15,16] with minor modifications.

Miscellaneous biochemistry

All SDS-PAGE analyses including Western blot and immunoprecipitation were performed under reducing condition according to published procedures [13,17] with minor modifications. In the experiments involving the analysis of total lysates, all cells were completely solubilized by boiling in 1% SDS, 100 mM Tris, pH