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Radicicol induces intracellular accumulation of glycan-deficient clusterin variant

Ilho Choi [a, c], Yumi Lee [a, c], Joong-Yeol Park [b, c], Youngsup Song [a, c], Eun-Ju Chang [a, c], Sang-Wook Kang [a, c, *]

[a] Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, Republic of Korea
[b] Department of Internal Medicine, University of Ulsan College of Medicine, Seoul, Republic of Korea
[c] Asan Institute of Life Sciences, Asan Medical Center, Seoul, Republic of Korea

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ABSTRACT

Proteostasis regulation using naturally occurring small molecules has been considered as a promising strategy for manipulating cancer sensitivity and therapy. Here, we identify a small molecule Hsp90 inhibitor radicicol that induces intracellular accumulation of cytotoxic clusterin variant. In the mechanistic basis, this variant proved to be a product disposed from the stressed ER. During this process, inhibitory effect of radicicol on protein degradation results in cytosolic accumulation of glycan-deficient clusterin variant that signals cell death. These results provide a therapeutic insight into the targeted proteostasis perturbation of clusterin as an anti-cancer strategy.

1. Introduction

Clusterin (CLU) is disulfide-linked heterodimeric secretory glycoprotein [1] whose expression is increased in various types of tumor and cancer cells by a variety of cancer treatments including hormonal manipulation, radiation and chemotherapy [2,3]. Hence, CLU has been believed to play protective role against cytotoxic stimuli [4,5]. In addition to secreted form of CLU, several intracellular isoforms have been discovered in the cells treated with ER stress inducers. These variants subsequently proved to be products that bypass the pathways to maintain protein homeostasis ("proteostasis") during ER stress. Intriguingly, this variant is rather cytotoxic than protective [6,7].

CLU seems to be especially sensitive to ER stress, as the protein undergoes multiple modifications with fine-tuned sequential engagement of processing and folding machinery in the ER [8]. During ER stress, misfolded CLU is realized by retrotranslocation machinery and disposed from the ER, and undergoes degradation in the cytosol by proteasome-dependent pathway [6,7]. In this context, perturbing degradation of misfolded CLU may be a potential strategy to accumulate cytotoxic CLU variant.

Many known naturally occurring or synthetic small molecules influence a part of proteostasis network. These compounds modulate protein quality control pathways including heat shock responses, ER stress responses and degradation machinery, which results in alteration of the stability, processing or localization of multiple "client" proteins [9]. Among them, small molecule heat shock protein 90 (HSP90) inhibitors are one of the most considerable classes of compounds for proteostasis perturbation in cancer cells [10,11].

In this study, we provide mechanistic insight into the generation of cytotoxic CLU variant by small molecule HSP90 inhibitors and discuss about therapeutic potential of targeted proteostasis perturbation with manipulating CLU turnover as an anti-cancer strategy.

2. Materials and methods

2.1. Cell culture

SH-SY5Y human neuroblastoma cells were kindly provided by Dr. S-Y Yoon (University of Ulsan College of Medicine, Seoul, Korea). Cells were grown in RPMI1640 supplemented with 10% FBS in 5% CO_2 at 37 °C.

* Corresponding author. Department of Biomedical Sciences, University of Ulsan College of Medicine, 88, Olympic-ro 43gil, Songpa-gu, Seoul 138-736, Republic of Korea. Fax: +82 2 3010 5307.
E-mail address: swkang@amc.seoul.kr (S.-W. Kang).

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2.2. Antibodies and reagents

Following antibodies were used in this study; anti-HA (Roche), anti-GRP78 (BD transduction laboratory), anti-HSP90 (Cell signaling), and anti-PrP, Sec61β, TRAPα (kind gifts from Dr. R. Hegde). Tunicamycin was purchased from Calbiochem. Radicicol, 17-AAG and geldanamycin were from Enzo Life Sciences. Chemicals were purchased from Sigma–Aldrich Korea.

2.3. Plasmid constructs

Constructs containing prion protein (PrP), prolactin (Prl), interferon gamma (Infγ), and osteopontin (Opn) signal sequences were kindly provided by Dr. R. Hegde (Laboratory of Molecular Biology, MRC, University of Cambridge, UK). DNA sequences encoding signal sequences and additional three amino acids were PCR-amplified with specific oligonucleotides (Cosmogenetech). Chimeric human clusterin (CLU) constructs containing foreign signal sequences (Fig. S5) were constructed as previously reported [12–14].

2.4. Biochemical analyses

Metabolic labeling of newly synthesized proteins followed by immunoprecipitation was performed as described [12,15]. Measurement of the relative rates of protein synthesis was accessed by quantitative analysis of [^{35}S] methionine (Roche) incorporated newly synthesized proteins in the cells as described [15]. Isolation of cytosolic and glycoproteins, and quantitation of newly synthesized **g**lycoproteins-to-**c**ytosolic proteins **r**atio (GCR) were analyzed as described [15]. Transfection, western blot, XBP-1 splicing, glycosidase assay, domain swapping assay and image analyses were performed as our previous report [7,12]. Following primers were used for the analysis of XBP-1 splicing; forward primer, 5′-TTACGAGAGAAAACTCATGGC-3′, reverse primer, 5′-GGGTCCAAGTTGTCCAGAATG-3′. In vitro transcription and translation were performed using previously published methods [16].

Exact times and conditions in each experiment are described in individual figure legends.

3. Results and discussion

3.1. Radicicol induces ER stress

This study was initially motivated by the observation that a small molecule HSP90 inhibitor radicicol derived from fungi [17] selectively alters the level of newly synthesized proteins in the ER (Fig. 1A) and induces cytotoxicity (Fig. S1). Given that similar effect has been observed in the cells treated with ER stress inducers [15], ER stress might be a reason for this observation. As expected, radicicol induced ER stress, as shown by a clear increase of GRP78 (BiP) protein level (Fig. 1B), an ER-resident chaperone and typical ER stress target protein, and spliced XBP-1 mRNA (Fig. 1C). On the other hand, the protein level of cytosolic chaperone HSP90 and unrelated protein TRAPα was unchanged (Fig. 1B).

As mentioned, several proteins synthesized at the ER are extremely sensitive to ER stress because of their requirement of the complex post-translational modifications including glycosylation, disulfide modification, and proteolytic cleavage. Clusterin (CLU) seems to be one such example [8]. This idea is supported by our previous observations demonstrating that ER stress induce intracellular accumulation of misfolded, immatured and misassembled CLU variant [7,12]. Of note, a similar form in mass to this ER stress-inducible CLU variant was newly synthesized in the cells treated with radicicol (Fig. 1D). Collectively, radicicol induces ER stress accompanied by impaired CLU biogenesis.

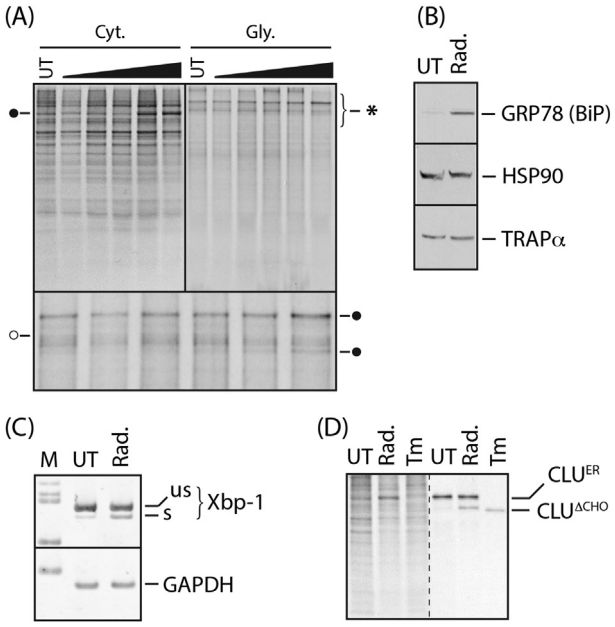

Fig. 1. Radicicol induces ER stress. (A) The rates of protein synthesis was accessed by analysis of newly synthesized cytosolic and glycosylated proteins. Cells were pretreated with radicicol (0, 0.016, 0.08, 0.4, 2, 10 μM) for 16 h hrs and pulse-labeled with [^{35}S] methionine (100 μCi/ml) for additional 15 min. Cytosolic contents (Cyt.) and glycosylated proteins (Gly.) were selectively extracted and isolated as described "materials and methods". Note, black and white circles indicate proteins dose-dependently increased and decreased, respectively. "UT" = untreated cells. The area indicated with "asterisk" was magnified (lower panel). (B) Expression of GRP78 (BiP) and HSP90 was monitored by Western blot analysis in fully solubilized cells treated with radicicol (10 μM) ("Rad.") for 16 h. Equal protein loadings were determined by TRAPα protein level. (C) ER stress-induced IRE-1 activation was monitored by XBP-1 mRNA splicing using specific primers for XBP-1 in the cells treated with indicated as (B). GAPDH was used as a reference for normalization. Note, "us" = unspliced form, "s" = spliced form. (D) Expression of CLU variant was monitored by metabolic-labeling followed by immunoprecipitation in the cells transfected with C-terminal HA epitope fused wild type human CLU construct (wtCLU-HA). Cells were treated with either radicicol (10 μM) or tunicamycin ("Tm") (5 μg/ml) for 10 h, and pulse-labeled with indicated as (A). "Total" = input, "IP" = pulldown with anti-HA antibody. Fully solubilized total cellular proteins were subjected to immunoprecipitation with anti-HA antibody. Note, "CLU^{ER}" = fully glycosylated ER form of CLU (~60 kDa), "CLU$^{\Delta CHO}$" = glycan-deficient CLU (~50 kDa).

3.2. Clusterin is a HSP90 client protein

In fact, CLU variant is generally undetectable when cells are treated with ER stress inducer alone, because of rapid and efficient degradation of misfolded CLU by proteasome-dependent pathway [7,12]. Intriguingly, in the cells treated with radicicol, CLU variant was accumulated and increased by dose-dependent manner even without proteasome inhibition (MG132) (Fig. 2A). This effect was also observed in the cells treated with the other HSP90 inhibitor geldanamycin and its analogue 17-AAG. At the same time, HSP90 inhibitors induced ER stress, as shown by an increase of GRP78 protein level (Fig. 2B). This result was further supported by an additional observation that similar level of GRP78 protein induction was shown in the cells even without CLU overexpression (Fig. S2).

Our biochemical analyses provided several pieces of valuable information about this CLU variant. First, this variant proved to be glycan-deficient as the product was resistant to glycosidases, Endo H and PNGase F (Fig. 2C) but identical in mass with CLU synthesized in the cells treated with glycosylation inhibitor tunicamycin (Tm) (Fig. 1D) and expressing mutant CLU lacking signal sequence (ΔSP) (Fig. 2B).

Second, this variant is unlikely to be nuclear CLU (nCLU) that previously defined as a spliced variant translated from the third

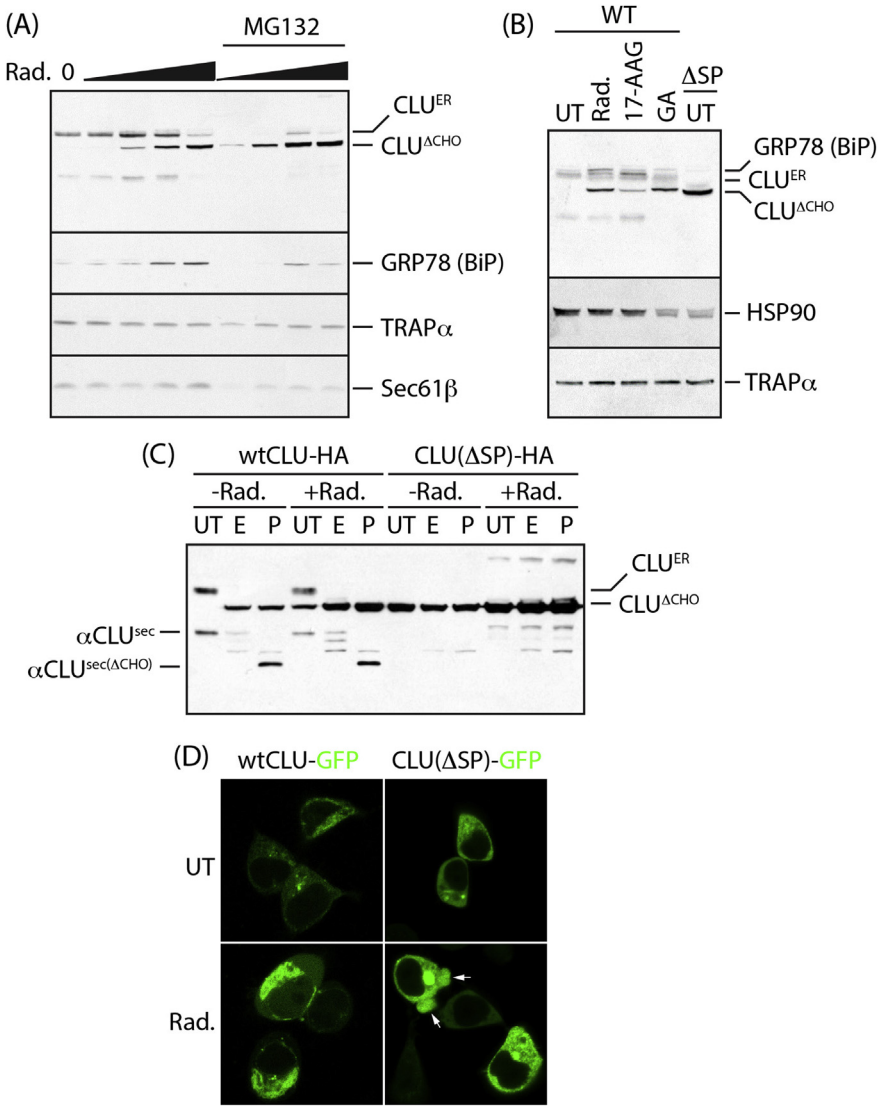

Fig. 2. HSP90 inhibitors induce intracellular accumulation of CLU variant. Expression of CLU variant was monitored by Western blot analysis under reducing condition. (A) Cells were transfected with wtCLU-HA construct and treated with radicicol as Fig. 1A in the presence or absence of MG132 (5 μM). (B) Cells transfected with wtCLU-HA or mutant CLU lacking signal sequence ("ΔSP") were treated with either radicicol (10 μM), 17-AAG (10 μM) or geldanamycin ("GA") (10 μM) for 16 h. Fully solubilized cells were subjected to Western blot analysis with anti-HA, GRP78, TRAPα, and Sec61β antibodies. (C) Generation of glycan-deficient CLU variant in the cells treated with radicicol was confirmed by glycosidase assay. Fully solubilized cells were diluted and digested with Endo H ("E") and PNGase F ("P"), and immunoblotted with anti-HA antibody. Note, "αCLU^{sec}" = fully glycosylated α-subunit of secretory CLU, "$\alpha CLU^{sec(\Delta CHO)}$" = glycan-deficient α-subunit of secretory CLU. (D) Subcellular localization of CLU was analyzed by confocal microscopy in the cells transfected with C-terminal GFP fused wild type ("wtCLU-GFP") and signal sequence-deficient mutant CLU ("CLU(ΔSP)-GFP") in the presence and absence of radicicol. Note, "arrow" = apoptotic bodies.

ATG of multiple in-frame ATG codons in human CLU [18,19], as judged by clear difference in mass with newly synthesized mutant CLU lacking glycan-moiety or signal sequence (Fig. S3). Third, the observation that any HSP90 inhibitors used in this study influences neither the protein level nor glycosylation of TRAPα mirrors that this variant is not a product generated by impaired global glycosylation efficiency (Fig. 2B and Fig. S2). At last, this variant turned out to be localized at the peri-nuclear region in the cytosol where the protein is likely to be more prone to aggregation than mutant CLU lacking signal sequence (Fig. 2C). Together, CLU biosynthesis is sensitive to Hsp90 inhibitors.

3.3. Radicicol has a long-term indirect effect on clusterin variant generation

Next, we were curious about the mechanism by which glycan-deficient CLU variant is generated. We monitored the translocation efficiency of CLU. This experiment is motivated by our previous observation that CLU contains relatively less efficient signal sequence and is a prominent substrate of a small molecule "cotransin" that selectively attenuates protein translocation into the ER [12] (Fig. S4).

First of all, we examined whether radicicol directly acts to CLU, using reticulocyte lysate-based cell free translation system. In the absence of ER-derived rough microsome (RM), in vitro translation of CLU resulted in the synthesis of a ~50 kDa product. This product is glycan-deficient CLU. In the presence of RM, ~60 kDa product that has been previously characterized as fully glycosylated CLU in the ER lumen [12] was newly synthesized, but instead ~50 kDa product was mostly disappeared. It is noteworthy that radicicol failed to remain glycan-deficient CLU product (~50 kDa) in the presence of RM (Fig. 3A). Whereas our metabolic labeling followed by immunoprecipitation showed a dose-dependent increase of newly synthesized CLU in the cells treated with radicicol (Fig. 3B). An indirect

effect of radicicol on CLU variant generation seems to be a reason for this paradoxical observation between in vitro and in vivo. This notion was further supported by the result showing that short-term treatment of radicicol failed to generate CLU variant (Fig. S4).

3.4. Radicicol perturbs clusterin biosynthesis at post-translocational level

Although clusterin variant turned out to be generated by an indirect effect of radicicol, we could not rule out the possibility that radicicol influences CLU translocation for the following reasons: first, CLU was sensitive to radicicol, second, radicicol induced ER stress, at last, protein translocation is selectively regulated by signal sequence-dependent manner during ER stress [15]. To determine whether translocational failure may be a reason for the generation of CLU variant, we engineered several chimeric CLU constructs in which native signal sequence of CLU was replaced with that of well-known secretory proteins of which translocation efficiency and susceptibility to ER stress have been characterized in our previous studies [12,15,20] (Fig. S5). The level of CLU variant expected to be paralleled by signal sequence susceptibility to ER stress. However, translocational attenuation seems not to be the reason for the generation of CLU variant, as shown by a similar level of newly synthesized CLU variants in pulse-labeled cells expressing chimeric CLU treated with radicicol (Fig. 3C). Our western blot analyses provide additional evidence to suggest that these CLU variants is not degraded but accumulated in the cells treated with radicicol (Fig. 3D). We conclude here that the translocational failure attributable to less efficient signal sequence is not the main reason for the generation of CLU variant.

3.5. Radicicol inhibits degradation of proteins disposed from the ER

It is noted that, even without radicicol, CLU variant is accumulated in the cells expressing Opn-CLU in which native signal sequence of CLU is replaced by highly efficient Opn signal sequence [20]. Indeed, the level of this variant is enhanced by radicicol treatment (Fig. 3D). Among the various possible reasons for this observation, the explanation proved to be perturbed-degradation of misfolded proteins disposed from the stressed ER (Fig. 4C). This could be shown by a couple of experiments.

The first experiment was motivated by the observation that cytosolic PrP mislocalized from the stressed ER is undetectable as this variant is efficiently degraded by proteasome-dependent pathway (Fig. S6). However, radicicol induces accumulation of glycan-deficient cytosolic PrP in the cells, even without proteasome inhibitor. In an agreement with Fig. 3, this effect turned out to be not dependent on signal sequence but mature region (Fig. 4B). Collectively, impaired degradation of mislocalized PrP from the stressed ER seems to be a reason for this observation.

An additional experiment to prove this idea was performed by measuring the relative rates of protein synthesis in the cells. Quantitative analysis of [^{35}S] methionine incorporation showed a decrease of newly synthesized cytosolic (digitonin fraction) and ER proteins (ConA fraction) (e.g., ~41 and ~34%, respectively) in the cells treated with radicicol. This seems to be general effect of ER stress [15]. However, combinatorial treatment of radicicol with kifunensin that inhibits retrotranslocation of misfolded proteins from the ER [21] selectively increased newly synthesized ER proteins up to ~156%, whereas cytosolic proteins were rather decreased by ~16%. In spite of similar GCR ratio in the cells untreated versus treated with either radicicol or kifunesin alone, their combinatorial treatment remarkably increased GCR ratio up to ~9.6 fold (Fig. 4A). This result indicates that radicicol inhibit degradation other than retrotranslocation of misfolded proteins from the stressed ER (Fig. 4C).

In conclusion, most important aim of this study is to identify the mechanism by which HSP90 inhibitor radicicol induces intracellular accumulation of glycan-deficient CLU variant. Major findings are two-fold. First, radicicol induces ER stress. Second, CLU is a HSP90 “client” protein.

In the mechanistic basis, radicicol-induced ER stress induces retrotranslocation of misfolded CLU. During this process,

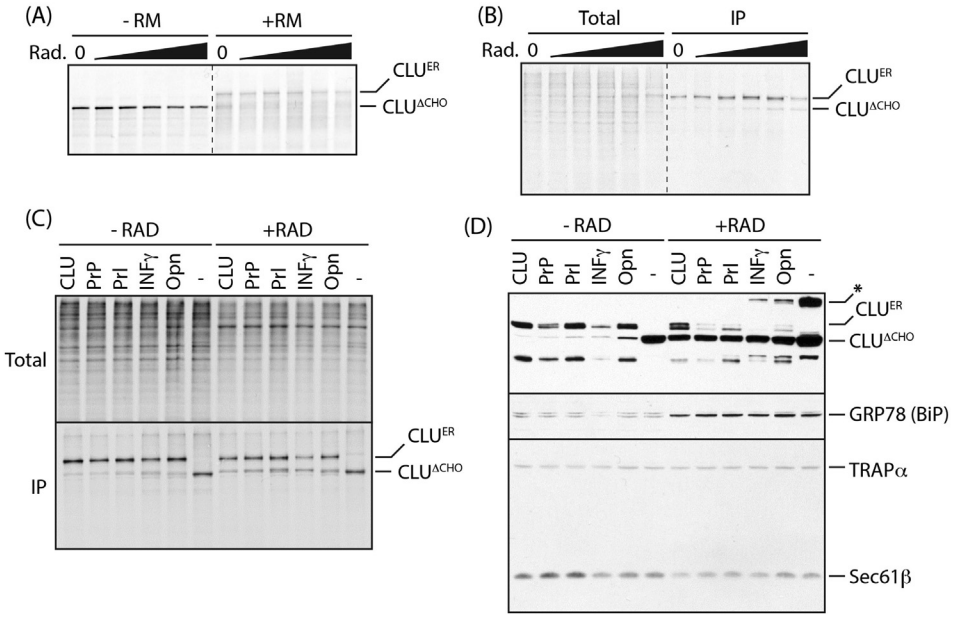

Fig. 3. CLU variant is generated by signal sequence-independent pathway. (A) Translocation efficiency of CLU was analyzed by in vitro translocation assay. wtCLU was translated in vitro in the absence (“-RM”; left panel) and presence (“+RM”; right panel) of rough microsome (“RM”) isolated from HeLa S3 cells. In this experiment, in vitro translation mixtures contain different amount of radicicol (0, 0.016, 0.08, 0.4, 2, 10 μM). Generation of newly synthesized CLU variant was monitored in pulse-labeled cells by immunoprecipitation. Following transfection of wtCLU-HA (B) or chimeric CLU constructs (Fig. S5) (C), cells were treated with radicicol as indicated in Fig. 1A (B) or Fig. 1D (C). Cells were pulse-labeled and subjected to immunoprecipitation with anti-HA antibody. (D) Accumulation of CLU variant was monitored in the cells prepared as described (C) by Western blot analysis.

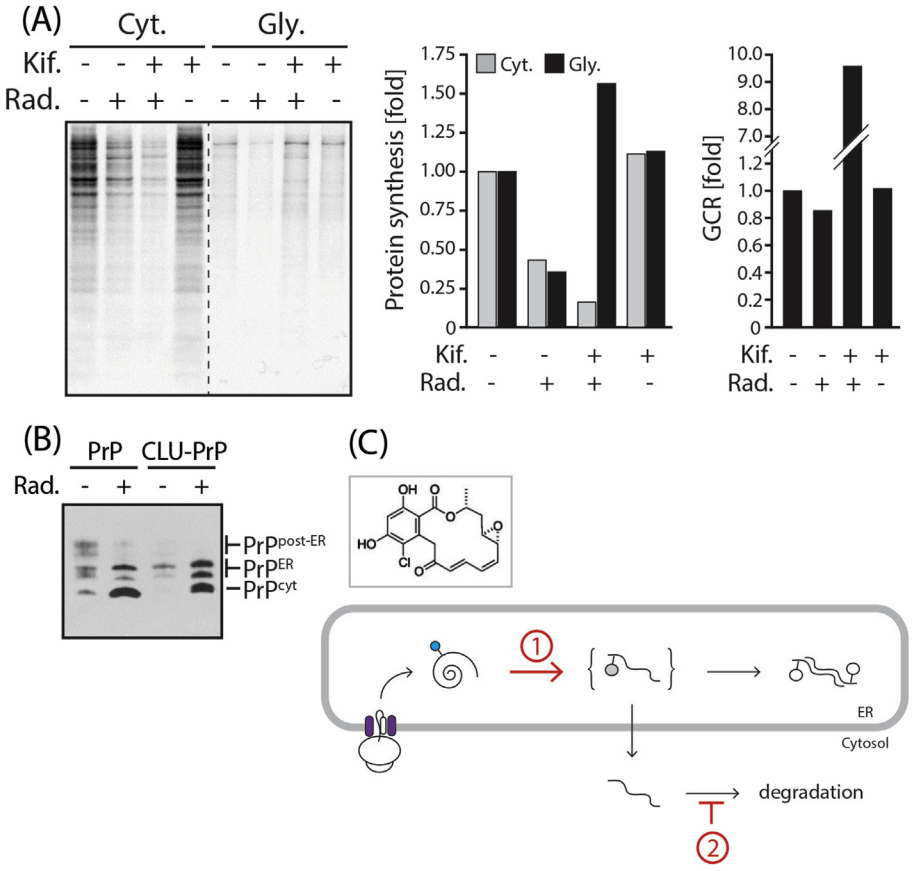

Fig. 4. Radicicol inhibits degradation of proteins disposed from the ER. (A) The rates of protein synthesis were quantitatively analyzed in pulse-labeled cells. Cells were treated with radicicol (10 μM) for 16 h followed by treatment of kifunensin (10 μM) ("Kif.") for 30 min. Following pulse-labeling with [^{35}S] methionine for additional 30 min, cytosolic contents ("Cyt.") and glycosylated proteins ("Gly.") were isolated as Fig. 1A (A, left panel). The graph was expressed as "fold induction" of radiolabeled proteins of each fraction (A, middle panel) and GCR ("**g**lycoproteins-to-**c**ytosolic protein **r**atio) (A, right panel) in the cells treated as indicated, relative to that in the cells without any treatments. (B) Upon radicicol treatment (10 μM for 16 h), generation of cytosolic PrP was monitored in the cells transfected with wtPrP and CLU-PrP by Western blot analysis with anti-PrP antibody. Note, PrP^{cyt} = glycan-deficient cytosolic PrP, PrP^{ER} = glycosylated ER forms of PrP, $PrP^{post\text{-}ER}$ = fully glycosylated post-ER form of PrP. (C) Potential mechanism of the generation of glycan-deficient CLU variant.

cytosolic HSP90 is unnecessary. Immediately after exposure to cytosolic side, misfolded proteins requires protein complex including co-chaperones, ubiquitin ligase CHIP, HSP70 and HSP90 for ubiquitination and degradation [22]. Given that CHIP binds to HSP90 C-terminal decapeptide [23], HSP90 inhibition possibly impairs ubiquitination of chaperone bound client proteins, particularly aggregation-prone proteins including PrP [15] and CLU [7]. This may be a plausible explanation for the observation that ubiquitinated CLU is undetectable upon radicicol treatment.

On the basis of physiological consequence, mislocalized CLU variant is apparently very minor species and degraded efficiently at the beginning. However, this variant is terminally misfolded, forms aggregates and turns cytotoxic by long-lasting proteostasis perturbation. This may be a more acceptable mechanism of slowly progressing diseases accompanied by CLU upregulation, such as neurodegenerative diseases, diabetes, and obesity.

In the therapeutic significance, given that CLU is upregulated by a variety of cancer treatments, combinatorial treatment with HSP90 inhibitors including radicicol may be a considerable therapeutic approach as an anti-cancer strategy.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.005.

Transparency document

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