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Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol

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Abstract

Clusterin is a usually secreted glycoprotein with chaperone properties. Recently, it has been suggested that clusterin isoforms reside in the nuclear and cytosolic compartments of human cell types, where they can influence various cellular programs including DNA repair, transcription and apoptosis. Several mechanisms have been proposed to explain this atypical location, including alternative transcription initiation and alternative splicing. However none of these have been unequivocally established as occurring in live cells. Here we provide direct experimental evidence that in live intact cells, under certain stress conditions, clusterin can evade the secretion pathway and reach the cytosol. This was demonstrated using several complementary approaches. Flow cytometry and selective permeabilisation of U251 cell membranes with digitonin allowed detection of cytosolic clusterin in stressed U251 cells. In addition, a stringent enzymatic assay reliant upon the exclusively cytosolic deubiquitinase enzymes, confirmed that clusterin synthesized with its hydrophobic secretion signal sequence can reach the cytosol of U251 cells. The retrotranslocation of clusterin is likely to occur through a mechanism similar to the endoplasmic reticulum (ER)-associated protein degradation pathway and involves passage through the Golgi apparatus. We also report that the ER-associated ubiquitin ligase Hrd1/synoviolin can interact with and ubiquitinate clusterin. The possible biological functions of these novel behaviours of clusterin are discussed.

Keywords

Stress, induced, retrotranslocation, clusterin, ApoJ, into, cytosol, CMMB

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Stress-induced retro-translocation of clusterin/ApoJ into the cytosol

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Running title: Clusterin is an intrinsic ERAD substrate

Summary

Clusterin is a usually secreted glycoprotein with chaperone properties. Recently, it has been suggested that clusterin isoforms reside in the nuclear and cytosolic compartments of human cell types, where they can influence various cellular programs including DNA repair, transcription and apoptosis. Several mechanisms have been proposed to explain this atypical location, including alternative transcription initiation and alternative splicing. However none of these have been unequivocally established as occurring in live cells. Here we provide direct experimental evidence that in live intact cells, under certain stress conditions, clusterin can evade the secretion pathway and reach the cytosol. This was demonstrated using several complementary approaches. Flow cytometry and selective permeabilisation of U251 cell membranes with digitonin allowed detection of cytosolic clusterin in stressed U251 cells. In addition, a stringent enzymatic assay reliant upon the exclusively cytosolic deubiquitinase enzymes, confirmed that clusterin synthesized with its hydrophobic secretion signal sequence can reach the cytosol of U251 cells. The retrotranslocation of clusterin is likely to occur through a mechanism similar to the endoplasmic reticulum (ER)-associated protein degradation pathway and involves passage through the Golgi apparatus. We also report that the ER-associated ubiquitin ligase Hrd1/synoviolin can interact with and ubiquitinate clusterin. The possible biological functions of these novel behaviours of clusterin are discussed.

Key words: protein translocation, clusterin, Apolipoprotein J, endoplasmic reticulum-associated protein degradation (ERAD), protein compartmentalization.

Introduction

Clusterin was initially identified as a secretory glycoprotein in ram rete testis fluid, capable of inducing the clustering of various cells (1). Since that time, clusterin has been found associated with a wide variety of molecules, and numerous biological activities. In humans, the protein is co-translationally transported to the lumen of the ER where, after being further glycosylated and cleaved to generate its α and β subunits in the Golgi apparatus, the protein is secreted (2). The secreted form of clusterin is a 75-80 kDa disulfide-linked heterodimeric glycoprotein (3). Clusterin has chaperone properties similar to the small heat shock proteins (sHSP). Its nonspecific binding to hydrophobic domains of partially unfolded proteins prevents stress-induced protein aggregation (2). Accordingly, clusterin gene transcription is highly reactive to proteotoxic stress (4, 5). Clusterin's increased expression in response to stress and its stabilizing interactions with unfolding proteins led to its classification as an extracellular chaperone (6, 7).

Determining the biological importance of clusterin has been complicated by the propensity of the protein to interact with a wide variety of molecules including lipids, which gave rise to the alternative name apolipoprotein J (8), amyloid proteins (9), components of the complement membrane attack complex (10, 11), immunoglobulins (12) and other molecules of clusterin (1). Moreover, while the clusterin gene encodes a secreted protein, reports have suggested that it occurs in the nucleo-cytosol continuum, where it has been proposed to influence, for example, DNA repair (13), transcription (14), microtubule organization (15) and apoptosis (13, 16, 17). A consensus has therefore emerged that clusterin's role is determined by the final location of the protein.

Various mechanisms have been proposed to explain how the usually secreted clusterin protein can reside in the nuclear and cytosolic compartments. Alternative initiation of transcription was proposed to be the mechanism yielding a 43 kDa clusterin isoform in the nuclear fraction of two epithelial cell lines, CCL64 and HepG2, treated with transforming growth factor beta (TGF-β) (18). A 49 kDa clusterin isoform discovered in the nuclear fraction of MCF-7 cells after ionizing radiation was proposed to be the result of alternative splicing (19). Cytosolic localization of clusterin may be especially important since, recently, a 60 kDa intracellular clusterin isoform was reported to confer upon cancer cells resistance to apoptosis and anti-tumoral treatments through antagonizing conformationally altered Bax in a human fibrosarcoma cell line (17); however, this study did not address how clusterin was diverted from its normal secretory pathway. Furthermore, clusterin has also been reported in the cytosol of rat adrenal pheochromocytoma PC12 cells after NGF treatment (15). Like the secreted form of clusterin, this isoform was approximately 80 kDa in size, which led to the hypothesis that secreted clusterin was internalised by cells (15). Notably, none of these studies performed sequence analysis of any of the intracellular isoforms, therefore it is not known whether differences in observed mass are due to alternative splicing or transcription initiation or simply due to differences in glycosylation. In addition, the methods used in some cases to identify the intracellular isoforms, such as cell fractionation, may have led to false conclusions. It is known that

clusterin binds to the nuclei of dead cells lacking an intact cell membrane (20). Disruption of live unfixed cells may lead to poorly glycosylated and/or uncleaved clusterin usually present in the ER/Golgi being released from these compartments and binding to nuclei, which would not normally occur in intact cells.

In this report, we provide evidence that clusterin can be retrotranslocated from the secretory system to the cytosol through a mechanism similar to endoplasmic reticulum (ER)associated protein degradation (ERAD). The founder observation which led to this study is the paradoxical decrease of mature clusterin protein in situations of cellular stress where the clusterin gene is upregulated, such as treatments with thapsigargin (21). This effect was also produced by a high extracellular concentration of KCl, which is known to induce stress gene expression in U251 astrocytic cells (22) and is naturally observed during ischemia, which itself is an inducer of clusterin expression (23). We show that in U251 cells, stress-induced clusterin is mainly targeted to the cytosol where it is, at least in part, processed by the ubiquitin-proteasome system. This novel behaviour of clusterin is discussed in relation to a potential role for the protein as an ER chaperone.

Results

Cytosolic degradation of mature clusterin after stress treatments

We recently showed that clusterin gene transcription was stimulated in the human glial cell line U251 after proteasome inhibition (24) and treatment with KCl (data not shown). Surprisingly, in spite of the increased clusterin gene transcription in these situations, clusterin protein levels were not markedly increased, particularly the mature cleaved form of clusterin. As shown in Figure 1 (right lanes), the major ER glycoform of the clusterin precursor (** around 70 kDa) remained unchanged following KCl treatment and was slightly increased after MG132 treatment. At the same time, clusterin secretion was markedly lowered (Figure 1, left panels). The cellular content of mature cleaved clusterin, as revealed by detection of the clusterin β subunit (* around 40 kDa), was reproducibly lowered after certain cellular treatments. It decreased after treatment with KCl but not with MG132 (Figure 1). Given that MG132 is a proteasome inhibitor, collectively, these results suggested that mature clusterin might be subject to proteasomal degradation and artificially stabilized by MG132. Since proteasomes are cytosolic structures, this in turn suggested that mature clusterin might be retrotranslocated to the cytosol.

In another experiment, the intracellular content of clusterin was compared in U251 cells either untreated or treated with KCl or thapsigargin (Tg), a classically used ER stress-stimulating agent which we showed to be less potent than KCl at inducing clusterin transcription (data not shown). KCl and Tg induced a decrease in the level of intracellular cleaved clusterin. These two treatments had opposing effects on the two forms of clusterin after a 48 h treatment (Figure 2A), decreasing the cleaved form and increasing uncleaved clusterin. These superficially paradoxical results are likely to be the product of the opposite influences of the different stresses on clusterin expression versus degradation. The decrease in the cellular content of cleaved clusterin caused by Tg was counteracted by MG132 treatment (Figure 2B, right panel), supporting the hypothesis that the stress-induced reduction in the intracellular level of cleaved clusterin results from proteasomal degradation. In some experiments, when using MG132, additional bands were detected between the major cleaved and uncleaved forms of clusterin (Figure 2B, arrow). Though these bands have not been formally identified, some of them could represent poly-ubiquitinated forms of clusterin. Such a possibility is strengthened by our previous finding that clusterin is a strong ubiquitination substrate when expressed directly in the cytosol (16). To determine if the additional clusterin immunoreactive bands could be polyubiquitinated clusterin, we tested if they could be enriched after FLAG-ubiquitin transfection and immunoprecipitation. Cells transfected with FLAG or FLAG-ubiquitin were treated with MG132 to stabilize ubiquitinated proteins by preventing their proteasomal degradation. FLAG-immunoprecipitated proteins were submitted to SDS-PAGE and probed with anti-β-clusterin antibody (Figure 2C). Some clusterin was recovered in the immunoprecipitate from lysate prepared from FLAGtransfected cells, which may result from the known interaction of clusterin with IgG (12). However, more clusterin and additional bands (indicated by arrows) were clearly detected specifically after transfection with FLAG-ubiquitin and with molecular weights distinct from the major forms of endogenous clusterin (Figure 2C). This result is consistent with covalent attachment of a variable number of ubiquitin molecules to clusterin.

The preferential degradation (Figure 2A, B) and presumptive polyubiquitination of cleaved clusterin (Figure 2C) led us to determine the cellular site of clusterin cleavage into α and β chains in U251 cells. We used the drug BFA, which disrupts ER-to-Golgi transport, to investigate this. Treatment of cells with BFA abolished clusterin processing since no cleaved clusterin could be detected even after overexposure of the immunoblot (Figure 2D). This result indicates that the cleavage generating the α and β subunits takes place in a distal part of the secretory system of U251 cells, most likely in the Golgi apparatus. The cleavage is likely to be carried out by furin-related proteases which are present in the trans-Golgi network and whose consensus recognition site RxxR/ (25) matches the cleavage site of clusterin. Hence, the processing of cleaved clusterin by the ubiquitin-proteasome system suggests that a fraction of clusterin reaching the cytosol is derived from the Golgi apparatus.

Flow cytometric analysis of the location of clusterin in stressed and unstressed cells after selective permeabilisation of the cell membrane

U251 cells, either untreated or stressed by exposure to Tg and MG132, were fixed and treated with digitonin to selectively permeabilize the cell membrane, leaving intracellular nuclear and ER membranes intact (26). For comparison, in other experiments, the cells were treated with Triton X-100 which permeabilizes both the cell and intracellular membranes. Intracellular antigens were then detected using specific antibodies and fluor-conjugated secondary antibodies. Owing to the differing membrane selectivities of the two detergents, it was expected that antigens located inside the ER would be detected in cells permeabilized with Triton X-100 but not in cells permeabilized with digitonin. This was verified for the antigen ERp29, which is exclusively located in the ER lumen (Figure 3A(i)). As expected, clusterin immunoreactivity was detected in untreated Triton-permeabilized U251 cells, presumably representing clusterin present in the ER/Golgi system (Figure 3A(ii), thin vs dashed line). Following treatment with Tg and MG132, the level of intracellular clusterin detected increased (Figure 3A(ii), thick vs thin line), consistent with the stimulating effect of MG132 on clusterin expression (24). This effect was also verified by confocal microscopy (Figure 3B). Lastly, clusterin immunoreactivity was detected in digitonin-permeabilized U251 cells only when the cells had been stressed with Tg and MG132 (Figure 3A(iii), thick line vs thin/dashed lines). Anti-clusterin antibody did not bind to unpermeabilized cells, whether they were untreated or treated with Tg and MG132 (data not shown). The result shown in Figure 3A(iii) represents a direct measure of clusterin that has accumulated in the cytosol in response to cell stress; the effect was highly reproducible and statistically significant ($p < 0.01$, Student's t-test using triplicate samples, data not shown). Confocal microscopic images presented in Figure 3B show that the endogenous clusterin content is moderately increased by Tg and strongly increased by MG132. The pattern appears more diffuse when the cells were treated with TG alone (panel (v)). However, microscopy alone does not allow a definitive determination of whether intracellular clusterin is present outside the ER and

Golgi apparatus.

Experimental assessment of clusterin retrotranslocation using the DUB assay

As an additional complementary approach to the flow cytometry experiments, we adapted an enzymatic test of cytosolic incorporation (5), based on the action of deubiquitinases (DUBs) which are exclusively cytosolic enzymes. A chimeric coding sequence was constructed. As represented in Figure 4A, it consists of a fusion at the C-terminal end of secreted clusterin with a ubiquitin moiety followed by GFP, yielding the protein Clu-Ub-GFP. In this system, the release of GFP from Clu-Ub-GFP can be used as a reporter of the action of DUBs and thus of the presence of the protein in the cytosol. In addition, to determine if the amount of clusterin present in the ER is a critical parameter for its cytosolic transfer, an ER retrieval motif KDEL was added (or not) at the C terminus of Clu-Ub-GFP to induce protein accumulation in the ER by preventing its secretion. U251 cells were transfected with Clu-Ub-GFP and Clu-Ub-GFP-KDEL and then treated (or not) with Tg, BAPTA-AM and MG132. After transfection, cells were analyzed by fluorescence microscopy. Tg induced reproducible changes of the ER shape into a foam-like structure (Figure 4B), previously observed in cases of defective ER membrane fusion (27). The KDEL motif led to an enrichment of fluorescence in the perinuclear region of the ER, but in all cases, as for the results of the confocal microscopy (Figure 3B), it was impossible to determine if a fraction of cytoplasmic GFP fluorescence was present in the cytosol outside of the ER. Hence, the fates of transfected Clu-Ub-GFP and Clu-Ub-GFP-KDEL were then examined by immunoblot analysis. We first tested clusterin immunoreactivity using an antibody reactive with the clusterin β-chain. As shown in Figure 4C, besides endogenous forms of clusterin (40 and 70 kDa), additional bands were detected in Clu-Ub-GFP-KDEL-transfected cells. The full length fusion protein was present (100 kDa), together with a minor band below 50 kDa and above 75 kDa. The concentration of these species was increased upon MG132 treatment and the apparent molecular weight of about 50 kDa is consistent with that of the clusterin β chain fused to an ubiquitin moiety but devoid of GFP, suggesting that DUB cleavage has occurred. The minor band between the 70 kD and 100 kD band could correspond to uncleaved clusterin fused to an ubiquitin but without GFP. The DUB cleavages were further examined through GFP immunodetection. In the immunoblot results shown in Figure 5, it was indeed possible to clearly visualize and quantify free GFP under certain conditions. We first examined the release of GFP from Clu-Ub-GFP-KDEL, to measure the extent of cytosolic translocation of this fusion protein. The level of free GFP appeared very low in basal conditions (Figure 5A, left lane). This result indicates that GFP does not impede translocation of the fusion proteins into the ER since the Clu-Ub-GFP fusion protein is fully confined inside the ER in the absence of stress treatment. In addition, we also used the Clu-Ub-GFP construct in *in vitro* transcription-translation experiments in the presence or absence of microsomes. The protein was cleaved in the absence of microsomes but was fully protected from DUB cleavage in the presence of microsomes (data not shown). Conversely, free GFP was clearly detectable in the presence of MG132 (Figure 5A, second lane from left). Tg, which stimulates ERAD, had little effect when added alone

(Figure 5A, third lane from left), but strongly stimulated the release of free GFP when used in conjunction with MG132. In this latter condition, the escape of Clu-Ub-GFP from the ER was substantial, as revealed by the ratio of free GFP over Clu-Ub-GFP (Figure 5A, right lane). To determine if the effect of Tg on the release of GFP was due to an ER stress or was the mere consequence of a disturbance of calcium homeostasis, we also tested the calcium chelator BAPTA which is known to affect calcium homeostasis without triggering an ER stress. When used alone, BAPTA had little or no effect on the amount of free GFP. BAPTA lowered the effect of the treatment with both Tg and MG132 (Figure 5B) and with MG132 alone (Figure 5C), in agreement with the inhibitory action of BAPTA on ER stress gene expression (28). These results confirm that the observed effects of Tg were mediated by an ER stress.

We also compared the GFP immunoreactive species obtained in the cells and in the culture medium, from Clu-Ub-GFP constructs carrying or not the KDEL ER retention signal (Figure 5C). As expected, KDEL prevented secretion (Figure 5C, right panel), leading concomitantly to an increase in the cellular content of GFP immunoreactive proteins (Figure 5C, left panel). In the absence of KDEL, two forms of recombinant proteins carrying the GFP epitope were detected in the medium. One of them was also detectable to a lower extent in cellular extracts and has a molecular weight of about 75 kDa, consistent with Clu(β-chain)Ub-GFP. The other one with a molecular weight of about 100 kDa is likely to represent uncleaved fully glycosylated Clu-Ub-GFP, as in Figure 4C, suggesting that the proteolytic maturation of clusterin was bypassed when Clu-Ub-GFP was over expressed. The size of Clu-Ub-GFP was slightly higher in the medium than in cellular lysates (Figure 5C, compare right and left panels), suggesting that the secreted protein is more intensively glycosylated. The absence of this 100 kDa putative fully glycosylated Clu-Ub-GFP from cellular lysates suggests that full glycosylation of clusterin is immediately followed by its secretion. No free GFP was detected in the culture medium. This result shows that the release of free GFP did not occur in the secretory pathway and firmly validates the DUB-based technique as a stringent reporter of cytosolic translocation. GFP immunodetection confirmed that double treatment with Tg and MG132 led to the strongest intracellular accumulation of free GFP (Figure 5C, left panel). The presence of the ER retrieval signal led to an increase in the total cellular content of expressed protein but did not significantly modify the ratio of free GFP to total GFP, suggesting cytosolic import of clusterin is more dependent on physiological conditions than on the clusterin concentration in the ER.

Interestingly, densitometric analysis of the different lanes in Figure 5C shows that in conditions where free GFP increased, the amount of mature cleaved clusterin, detected as Clu(beta-chain)-Ub-GFP, was reduced in the cells (Figure 5D, left panels) and, more obviously, in the culture medium (Figure 5D, right panels). This inverse correlation suggests that upon ER stress, clusterin cleaved in the Golgi apparatus and normally destined for subsequent secretion is retrotranslocated back to the cytosol. This observation is consistent with the previous results suggesting preferential processing of the cleaved form of clusterin by the ubiquitin-proteasome system (Figure 2). We also examined, using the DUB assay with a U251 cell clone stably expressing Clu-Ub-GFP, the effects of two other ER stress agents: tunicamycin (Tun) and BFA. In contrast to Tg, in addition to inducing ER stress, these agents also disrupt the transport of proteins from the ER to the Golgi, by inhibiting glycosylation (Tun) or vesicular trafficking (BFA). Figure 5E shows that the inhibition of glycosylation by Tun led to a striking mobility shift of Clu-Ub-GFP. The band corresponding to Clu(betachain)-Ub-GFP could not be detected when using Tun and BFA, reflecting the absence of passage of Clu-Ub-GFP into the Golgi apparatus. Consistently, contrary to the effects of Tg, Tun and BFA did not increase the level of free GFP. This result further suggests that in the case of clusterin, the main retrotranslocation substrate derives from the Golgi apparatus. This conclusion is further supported by the effects of Tg in this model (inspect the three left lanes in Figure 5E). Relative to either control or MG132 alone treatments, Tg induced an increase in the amount of free GFP (an indicator of the level of retrotranslocation) while concomitantly decreasing the level of Golgi-derived Clu(beta-chain)-Ub-GFP.

Hrd1/synoviolin is a candidate ubiquitin ligase for clusterin

Protein degradation in ERAD is generally ensured by ER-associated ubiquitin ligases. Considering the intrinsically disordered structure of clusterin (29, 30), we tested the RING finger E3 ubiquitin ligase Hrd1/synoviolin, because it had been shown in yeast to bind preferentially to misfolded proteins (31) and is anchored in the ER membrane. Pull down experiments (Figure 6A) show that clusterin can bind *in vitro* to a GST-Hrd1 fusion protein (in which the hydrophobic Hrd1 membrane insertion domain has been deleted). As controls, we verified that clusterin does not bind to GST alone and that GST-Hrd1 does not bind to luciferase. We then tested the capacity of Hrd1 to ubiquitinate clusterin *in vivo*, by overexpressing Hrd1 in the absence of stress. COS-7 cells were transfected with various combinations of plasmids encoding cytosolic clusterin-GFP (Δss-Clu-GFP), Hrd1/synoviolin and FLAG-ubiquitin. Proteins covalently ligated to FLAG-ubiquitin were then immunoprecipitated using the M2-anti-FLAG resin and tested by anti-GFP immunoblotting. As shown in Figure 6B, FLAG-ubiquitin-linked clusterin forms were clearly detected and their abundance was increased in cells transfected with Hrd1/synoviolin. We verified that under the same experimental conditions, GFP alone was not conjugated to FLAG-ubiquitin (data not shown). These results indicate that Hrd1 overexpression increases ubiquitination of cytosolic clusterin. Hrd1/synoviolin is thus a good candidate E3 ubiquitin ligase for clusterin once in the cytosol.

Discussion

Using two very different approaches, we have shown for the first time that clusterin can be retrotranslocated from the secretory system to the cytosol. This novel behaviour of clusterin could have fundamental biological consequences and may provide a mechanistic explanation for the steadily accumulating reports of intracellular clusterin. Recent studies have shown that unambiguously assessing protein retrotranslocation is a complicated task subject to numerous artefacts. The classical approaches used to assess the retrotranslocation of proteins, microscopy and cell fractionation, suffer from several drawbacks. Microscopy analyses are hindered by the changing and blurred outline of the ER. In addition, fluorescence is a nonlinear signal with threshold effects, so that low doses of fluorescence diluted in the cytosol cannot be detected when compared to that confined inside the ER, as discussed in (32). Cell fractionation techniques also pose difficulties because the membrane continuum between nucleus and perinuclear ER prevents complete separation of the different cellular compartments and has the potential to produce misleading results. For example, in the present case, minor contamination of the cytosolic fraction with some ER content during cell lysis could easily be incorrectly interpreted as physiological escape from the ER. These drawbacks are circumvented by the newer, complementary approaches used in this study. The selective membrane permeabilization technique offers the possibility to study endogenous proteins without altering their biosynthesis. Furthermore, owing to strict compartmentalization of the DUB enzymes

in vivo, the DUB assay provides an additional stringent tool to track the location of proteins in intact cells. DUBs have already been used to assess the efficacy of secretion signals for co-translational translocation into the ER (33); here we show here that they are also useful for studying the reverse pathway of retrotranslocation.

Another level of complexity results from the difficulty in distinguishing between protein retrotranslocation and aborted translocation into the ER lumen after translation. For example, this issue has led to debate about whether the prion protein is subject to ERAD (34, 35); it now appears likely that cytosolic accumulation of the prion protein results from the relative weakness of its secretion signal peptide (32). The same possibility has also recently emerged in the case of the ER lumenal protein calreticulin. It has been estimated that 5% of ER calreticulin can retrotranslocate to the cytosol, which would explain the multiple proposed functions of this protein in the nucleo-cytosol continuum (36). However, the cytosolic pool of calreticulin has also been attributed to an aborted translocation of the same fraction (5%) of nascent calreticulin into the ER lumen (37). The strong ERAD activity of clusterin is more clearly evidenced, thanks to complementary observations. The fact that cleaved endogenous clusterin appears, under certain conditions, to be subject to polyubiquitination (Figure 2C) and proteasomal degradation (Figures 1 and 2A), strongly suggests that it passed through the secretory pathway before reaching the cytosol. The behaviour of Clu-Ub-GFP also supports this view, since free GFP accumulated in the cells as the level of Clu(beta-chain)-Ub-GFP decreased, both in the cells and in the medium (Figure 5C, D, E), suggesting that a substantial fraction of retrotranslocated clusterin in U251 cells in our experimental conditions is mature, proteolytically cleaved in the Golgi apparatus, and fully glycosylated clusterin. This finding suggests that clusterin is at least in part perceived as an ERAD substrate at the level of a distal Golgi checkpoint. Clusterin may thus be a substrate for a subset of ERAD pathways termed ERAD-L, according to the classification of Vashist and Ng, based on the location of the protein (38).

The high capacity of cleaved clusterin for retrotranslocation does not preclude the possibility that uncleaved clusterin may also undergo this process but be a poorer ubiquitination substrate (Figure 2C). This scenario would be in agreement with previous reports which have claimed that a 60 kDa intracellular form of clusterin, likely to correspond to a poorly glycosylated ER clusterin precursor, interacts with intracellular molecular targets such as intracellular domains of TGF beta receptors (18) and activated Bax (17). Ubiquitination of retrotranslocated clusterin may be carried out, at least in part, by Hrd1/synoviolin; our results show that this ligase binds to and mediates ubiquitination of clusterin *in vitro* and that its over-expression enhances ubiquitination of clusterin *in vivo* (Figure 6). We showed that overexpression of Hrd1/synoviolin led to the accumulation of ubiquitinated forms of cytosolic clusterin in absence of cellular stress. Hrd1/synoviolin is an ER stress-regulated gene, containing a typical tripartite ER stress element (ERSE): CCAAT N_4 GGC N_2 CCACG, identical to those present in the archetypal ER stress-responsive gene BiP/GRP78 (39). Expression of Hrd1/synoviolin is strongly upregulated in response to Tg, hence, overexpression of ubiquitin ligases like Hrd1 is likely to explain why Tg caused a decrease in intracellular clusterin levels (Figure 2A and B).

The fact that at least a substantial fraction of retrotranslocated clusterin is subject to proteasomal degradation could appear as a puzzling and energy consuming phenomenon of uncertain biological significance. However, this process could relate to the chaperone activity of clusterin. Considering its high affinity for slowly aggregating proteins, one could hypothesize that clusterin may participate in the clearance of aberrantly folded proteins from the secretory system. It was recently proposed that clusterin may stabilize in solution damaged extracellular proteins and facilitate their cellular uptake and subsequent lysosomal degradation (7). The current work suggests that clusterin may also facilitate the removal of aberrantly folded proteins from the secretory system by escorting them to sites of proteasomal degradation. These two complementary activities could, for example, explain the capacity of clusterin (acting in conjunction with ApoE) to suppress the formation of extracellular amyloid deposits (40). Work is in progress to determine the influence of the forced expression of clusterin on the clearance of conditionally unfolded proteins from the secretory system.

ERAD was originally identified as a cellular activity aimed at discarding misfolded proteins from the ER, a cellular compartment devoid of proteolytic machineries. This system has subsequently been shown to also process a number of unstressed proteins, such as certain plant and bacterial toxins, whose molecular targets are cytosolic. The capacity of toxins to exploit the ERAD pathway to enter cells is believed to depend on their intrinsically disordered structure, which mimics an unfolded protein (41). The same possibility could be proposed for clusterin, which also has substantial regions of structural disorder (29). A notable feature of bacterial or plant toxins is their capacity to evade degradation once in the cytosol, thus allowing them to exert their cytotoxic activity. These toxins thus demonstrate that, contrary to its name, the ERAD system is not always coupled to protein degradation. In the same way, we cannot

exclude the possibility that a fraction of retrotranslocated clusterin escapes degradation once in the cytosol, and can interact with cytosolic molecules such as Bax (17). Zhang et al. found that by binding to conformationally altered Bax in the cytosol, clusterin interfered with its pro-apoptotic activity in cancer cells, resulting in cellular resistance to chemotherapeutic drug-mediated apoptosis. Thus, finding a way to inhibit clusterin retrotranslocation might in the future provide a new approach to render cancer cells more susceptible to pro-apoptotic therapies.

Materials and Methods

Plasmids

Expression plasmids for Clu-Ub-GFP and Clu-Ub-GFP-KDEL were constructed from the I.M.A.G.E. clone N°4150452 (pCMV-SPORT6-Human apolipoprotein J), in which ubiquitin-GFP and KDEL cassettes were inserted in frame. Briefly, a unique *Nar*I site was inserted at the end of the clusterin ORF by PCR-mediated mutagenesis using the Quickchange site directed mutagenesis kit (Stratagene) and complementary primers corresponding to the sequence 5'-GCACCGGGAGGAGGGCGCC**TgA**GATgTgGATg-3' (*Nar*I site is underlined and the stop codon of the clusterin ORF is bold). This site was used for insertion of a ubiquitin-EGFP fusion ORF obtained by PCR from the pDG268 plasmid (a gift from Dr. Doug A. Gray, Ottawa Regional Cancer Centre, Canada) and primers including the *Nar*I recognition site. To generate Clu-Ub-GFP-KDEL, the KDEL coding sequence was inserted at the C-terminus of GFP by PCR-based mutagenesis using complementary primers corresponding to the sequence 5'-CTgTACAAGGGCGCCAAAGATgAGTTg**TgA** GATgTgGATgTTg-3' (KDEL coding region is underlined and the stop codon is bold). Two expression vectors for Hrd1/synovioline were used in this study: the I.M.A.G.E. clone N°5211742 (pCMV-SPORT6-Human Hrd1) and pCDNA3-FLAG-full length synoviolin (a gift from Dr. Naoko Yagishita, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan). The Δss-Clu-GFP construct, encoding a full length clusterin sequence (minus the secretion signal peptide) which is expressed in the cytosol fused to GFP, has been described previously (16).

Cell Culture, transfection and treatments

The human astrocytoma cells U-251 MG were maintained with a 5% (v/v) CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 1 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (Gibco/Life Technologies, Paisley, UK). Plasmids were transfected using the calcium phosphate coprecipitation method. Cells were treated or not for the indicated times with 5 µM MG132, 2.5 µM thapsigargin (Tg) or 2.5 µg/ml Tunicamycin (Sigma-Aldrich, St. Louis, MO, USA). Tg inhibits the ER calcium pumps mediating calcium reuptake, which leads to dysfunction of calcium-dependent ER chaperones and ultimately to the accumulation of misfolded protein inside the ER. Tg thus leads to an ER stress and an upregulation of the genes encoding ERAD machineries (42). In some experiments, cells were also treated with 30 µM of the free cytosolic calcium chelator O,O'-Bis(2-aminophenyl)ethyleneglycol N,N,N',N'tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) or 30 µM Brefeldin A (BFA, Sigma-Aldrich, St. Louis, MO, USA), a metabolite which disrupts the structure and function of the Golgi apparatus.

Fluorescence and confocal microscopy

24 h after transfection, cells were rinsed in phosphate buffered saline (PBS: 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.4) and fixed for 15 min in 3% paraformaldehyde, then stained with 10 ng/ml DAPI in PBS and mounted in Mowiol 4-88 (Calbiochem, chemical formula not available). The GFP fluorescence in fixed cells was detected using epifluorescence microscopy (Olympus AX70, MWB filter for GFP (excitation 450-480 nm, emission above 515 nm) and AnalySIS software). For confocal microscopy, cells were grown on glass coverslips, fixed by 30 min incubation in 4% (w/v) paraformaldehyde in PBS at room temperature, and permeabilized by incubation on ice in 0.5% (v/v) TX-100 in PBS. Permeabilised cells were then incubated for 30 min on ice with either G7 anti-clusterin monoclonal antibody (as undiluted hybridoma cell culture supernatant) or an isotype-matched (IgG1k) control antibody (DNP-9; also as undiluted hybridoma cell culture supernatant). These antibodies have been described before (2). Bound primary antibody was detected by incubating cells with 2.5 µg/ml goat-anti-mouse Ig-Alexa 488 conjugate (Molecular Probes. Invitrogen, Cergy Pontoise France) in PBS for 30 min at 4˚C. Cells were resuspended in 500 µl of PBS and viewed with an SP confocal microscope (Leica, Sydney, Australia) with excitation at 488 nm and emission collected at 500 – 550 nm.

Immunoblotting

Cells were scraped from the culture surface and lysed for one hour on ice in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% (v/v) NP40, pH 8.0) supplemented with Complete protease inhibitor cocktail (Roche Diagnostics S.A. Meylan, France) and 3 mg/ml N-ethyl-maleimide, an inhibitor of thiol proteases. After a 30 min centrifugation at 14,000 g, proteins in the clarified lysates were separated by reducing 7.5% or 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Primary antibodies: anti-β-clusterin (M18 sc-6420, Santa Cruz Biotechnology, Inc. Santa Cruz, California 95060 USA) and anti-GFP (ab290, Abcam, Paris France), were revealed by enhanced chemiluminescence using horseradish peroxidase-conjugated IgG, dilution 1:5000 (GE Healthcare Europe GmbH. Saclay. Orsay, France). In some experiments, samples of culture medium (15 µl) were also analysed by immunoblotting as described above. Density plot profiles were obtained using the ImageJ program (National Institutes of Health).

GST pull-down of in vitro translated proteins

GST and GST-ΔTM synoviolin/Hrd1 were expressed in *Escherichia coli* BL21 from pGEX plasmids. Bacteria were induced with 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG Sigma) for 3 h at 20°C. Recombinant proteins were purified with glutathione–Sepharose beads (GE Healthcare Europe GmbH. Saclay. Orsay, France). Equivalent amounts of GST fusion proteins, as estimated by SDS–PAGE analysis, were incubated overnight at 4°C with [^{35}S]methionine-labeled human clusterin or firefly luciferase, produced by the T7/SP6 TNT-coupled transcription/translation system (Promega. Charbonnières France), in 200 µl of binding buffer (1xPBS, 0.5% (v/v) Triton X-100). The beads were washed five times with 1 ml of binding buffer. The bound proteins were eluted with SDS sample buffer, fractionated by SDS–PAGE and visualized by autoradiography.

In vivo ubiquitination assays

Cells were transfected with expression plasmids encoding ubiquitin fused to the FLAG epitope at its NH2 terminus, or FLAG alone. Proteins conjugated to FLAG-ubiquitin were then immunoprecipitated using anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO, USA) and the presence of clusterin in the immunoprecipitates was assessed by immunoblotting. For testing the involvement of HrdI/synoviolin in clusterin ubiquitination (Figure 6), COS-7 cells were also transfected with a plasmid encoding cytosolic clusterin, devoid of the secretion signal peptide and fused to GFP (ΔSS-Clu-GFP). COS7 cells were chosen for their capacity to replicate plasmids carrying the SV40 replication origin, for high level expression of transfected genes. The detailed ubiquitination protocol was as follows. $2x10^6$ transfected COS-7 cells were incubated for 6 h in presence of 5 µM MG132 and then lysed for 1 h on ice in 250 µl of lysis buffer. The lysates were centrifuged at 20,000 g for 30 min at 4°C. Soluble proteins were quantified using the DC protein assay (Biorad) and 10 µg was used as input for immunoblotting analyses. Equivalent amounts of soluble proteins were incubated with anti-FLAG M2 affinity gel for 4 h at 4°C. After extensive washing in Trisbuffered saline (TBS: Tris-Cl pH 7.5, NaCl 150 mM), 1% Triton X-100 and then in TBS only, the immune complexes were analyzed by immunoblotting as described above, using either anti-GFP (Figure 6) or anti-clusterin (Figure 2C) antibodies. To determine if endogenous clusterin can be ubiquitinated in U251 cells (Figure 2C), no clusterin expression vector was added.

Flow cytometry

U251 cells, untreated or exposed to Tg and MG132 as described above, were fixed by 30 min incubation in 4% paraformaldehyde in PBS at room temperature, then permeabilized by a 10 min incubation on ice in 10 µM digitonin in PBS or a 30 minute incubation on ice with 0.5% (v/v) Triton X-100 in PBS. Digitonin selectively permeabilizes the cell membrane (Lorenz et al., 2006) while Triton X-100 permeabilizes cell and intracellular membranes. Permeabilized cells were then incubated on ice for 30 min with G7 anti-clusterin monoclonal antibody or DNP-9 control antibody (both as hybridoma culture supernatants). Bound primary antibody was subsequently detected by incubating cells on ice for 30 min with goat-anti-mouse Ig-Alexa 488 conjugate (Molecular Probes. Invitrogen Cergy Pontoise , France). In other similar experiments, we confirmed that digitonin permeabilized the cell membrane but not the ER by incubating cells with an antibody probe for ERp29 (an antigen found exclusively in the ER lumen; (43)). Cells were incubated on ice for 30 min with rabbit anti-ERp29 antiserum (Abcam, Paris France), or pre-immune rabbit serum, both diluted 1 in 500 in 1% bovine

serum albumin in PBS (BSA/PBS), followed by sheep-anti-rabbit-FITC conjugate (Silenus Laboratories, Vic. 3122 Australia; diluted 1 in 50 in BSA/PBS). Flow cytometry was performed with a Becton Dickinson LSR II. All samples were supplemented with 1 µg/ml propidium iodide (PI) immediately prior to analysis to stain the nuclei of permeabilized cells. Excitation was at 488 nm and emissions were collected at 515 +/-20 nm (Alexa 488) and at 695 +/-40 nm (PI). Electronic gating was used to collect signals only from permeabilized (PI+) cells. Data was collected using FACS Diva software (v4.0; Becton Dickinson, Sydney) and analysed using FloJo v6.4.1 (Treestar Inc., Ashland, OR 97520 USA).

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Figure legends

Figure 1: Immunoblot analysis of secreted and intracellular endogenous clusterin after exposure to KCl and proteasome inhibition. The culture medium was changed 20 hours after cell spreading. U251 astrocytic cells were then treated with extracellular KCl (panel A) or with the proteasome inhibitor MG132 (panel B). At subsequent time points indicated on the figure, cell lysates (prepared as described in Materials and Methods) and aliquots of culture medium were analysed for clusterin content. Mature cleaved form (*), uncleaved intracellular clusterin precursor (**). After SDS–PAGE, the membrane was stained with Ponceau S solution to assure equal protein loading (P). The results shown are representative of three independent experiments.

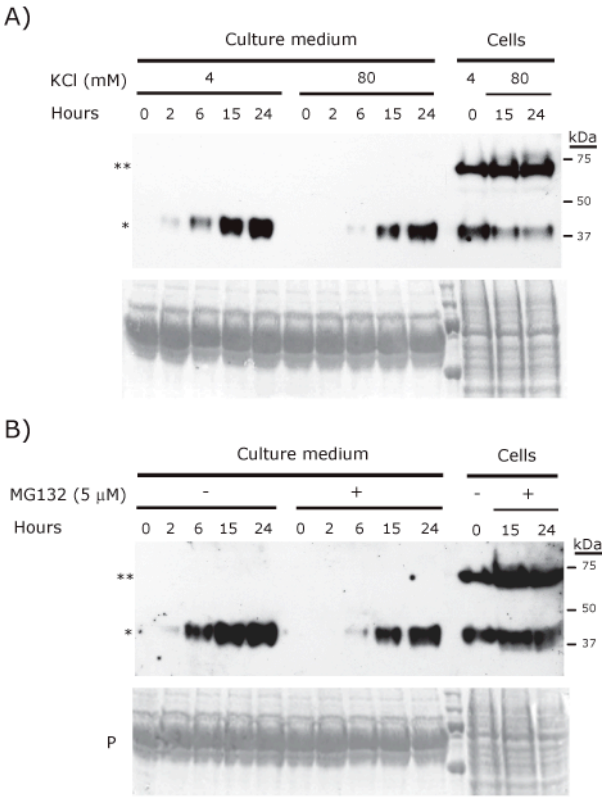
Figure 2. Western blot analysis of endogenous clusterin in U251 cells treated with different stresses. A) Treatments with KCl or Tg led to a decrease in the intracellular content of cleaved clusterin (*). B) MG132 treatment can counteract this effect of Tg. U251 cells were treated for 16 h or 24 h with Tg, MG132 or both. Lower panels (P) indicate Ponceau staining of the membrane prior to immunoblotting. C) U251 cells were transfected with FLAG or FLAG-ubiquitin and then treated with MG132 for 16 hours. Proteins were then extracted, immunoprecipitated or not (input), using anti-FLAG antibody-coupled agarose (IP) and resolved by SDS-PAGE, western blotted and then probed using anti-clusterin antibody. The heavy chain of anti-FLAG immunoglobulin is visible after Ponceau staining (lower panels, P). D) Effect of BFA on intracellular clusterin. Proteins were extracted from U251 cells treated or not with BFA and probed with anti clusterin β chain antibodies by immunoblotting. Lower panels (P) indicate Ponceau staining of the membrane prior to immunoblotting. For A) and D), the antibodies directed against the β subunit of clusterin revealed mature cleaved clusterin (*, β subunit around 40 kDa) and poorly (**, about 70 kDa) or fully (***, about 80 kDa) glycosylated uncleaved clusterin. The vertical bars and arrows shown to the right of the panels B and C indicate possible covalent attachment of a variable number of ubiquitin molecules to clusterin.

Figure 3. Flow cytometry and confocal microscopic analyses of U-251 cells. A) Histogram overlays showing green fluorescence of fixed U-251 cells. (i) Untreated cells permeabilised with digitonin and stained with either pre-immune rabbit serum (dashed line) or anti-ERp29 antiserum (thin line), or permeabilised with Tx-100 and stained with anti-ERp29 antiserum (thick line). Tx-100-permeabilized cells stained with rabbit pre-immune serum had a fluorescence histogram almost identical to the dashed line (data not shown). (ii) & (iii) Cells were permeabilised with either Tx-100 or digitonin (indicated in the panels); untreated cells were stained with control antibody (DNP-9, dashed line) or anti-clusterin antibody (G7, thin line); cells treated with MG132 + Tg were stained with G7 (thick line). For clarity, the fluorescence of cells treated with MG132 + Tg and stained with DNP-9 (which was in all cases almost identical to untreated cells stained with the same antibody) is not shown. The results shown are representative of 3 independent experiments. B) Confocal microscopy images of fixed and permeabilized U-251 cells. (i) Transmission image of U-251 cells; (ii) untreated cells stained with control DNP-9 antibody; (iii)-(vi) cells stained with anti-clusterin G7 antibody following (iii) no treatment, or treatment with (iv) MG132, (v) Tg or (vi) MG132
+ Tg. Scale bar: 10 μm.

Figure 4. DUB-based enzymatic assay for the cytosolic transfer of clusterin. A) Scheme of the chimeric sequence encoding a fusion protein made of, from the amino to the carboxyterminus, the full length clusterin precursor (α-clusterin hatched cassette and β-clusterin dotted cassette) with its secretion signal (SS, black cassette), an ubiquitin moiety (black disk), and enhanced GFP (vertical hatches). In one construct, the ER retrieval motif KDEL was added at the carboxy terminal end of the whole protein. B) Fluorescence microscopy analysis of U251 cells expressing Clu-Ub-GFP or Clu-Ub-GFP-KDEL. After transfection, the cells were either untreated (Cont.) or treated with Tg + MG132. Photographs were exposed for 400 ms for Clu-Ub-GFP-KDEL and 2 s for Clu-Ub-GFP. Nuclei were stained with DAPI. Scale bar: 10 μm. C) Immunoblot analysis (using anti β-clusterin antibodies) of total protein extracts from U251 cells, transfected or not with Clu-Ub-GFP-KDEL and treated or not with MG132. Interpretation of the identity of the different bands is indicated at the right, corresponding to the schematic shown in A. These results are representative of 3 independent experiments.

Figure 5. Immunoblot analysis of different protein forms incorporating GFP in U251 cells transfected with Clu-Ub-GFP constructs. A) GFP-immunoreactive bands present in U251 cells transfected with Clu-Ub-GFP-KDEL and then either untreated or treated with MG132, Tg, or a combination of MG132 + Tg. B) GFP-immunoreactive bands present in U251 cells transfected with Clu-Ub-GFP-KDEL and then either untreated or treated with MG132 + Tg, with BAPTA-AM, or with all the three compounds together. C) GFPimmunoreactive bands present in either cell lysates (left panel) or the surrounding culture medium of the same cells (right panel). The upper band corresponding to full length Clu-Ub-GFP differed in size between cells and culture medium, suggesting higher glycosylation of the secreted protein. D) Densitometry plots of lanes of the gel shown in panel C. Asterisks indicate the molecular species whose respective intensity is modified by Tg. E) GFPimmunoreactive bands present in U251 cells stably transfected with Clu-Ub-GFP and either untreated (Cont) or treated with MG132 and either Tg, Tun or BFA. These results are representative of three independent experiments. Interpretation of the identity of the different bands corresponds to the schematic shown in Figure 4A.

Figure 6. Hrd1/synoviolin is a candidate ubiquitin ligase to act on clusterin. A) Pull down assay of the interaction between Hrd1 and clusterin. Recombinant GST or GST-ΔTM Hrd1/synoviolin (with the transmembrane domain deleted to ensure solubility) were incubated with *in vitro* translated [^{35}S]-labelled full length clusterin (Clu) or luciferase (Luc) and stable complexes were purified from the incubation medium (supernatants; Sup.) by GST pull down with glutathione-Sepharose (pellets; Pell.). B) *In vivo* ubiquitination of clusterin-GFP is stimulated by overexpression of Hrd1/Synoviolin. COS-7 cells were co-transfected with the indicated combinations of expression plasmids: FLAG alone (FLAG), ubiquitin fused to the FLAG epitope at its NH2 terminus (FLAG-Ubiquitin), cytosolic clusterin devoid of the secretion signal peptide and fused to GFP (ΔSS-Clu-GFP), GFP alone and Hrd1. FLAGubiquitin protein conjugates were purified with anti-FLAG M2 affinity gel. Proteins from the input protein extracts (Input) and immunoprecipitates (IP FLAG) were separated by SDSPAGE and analysed by anti-GFP-immunoblotting. Ponceau red staining (P) is shown to allow evaluation of the total amount of loaded proteins, the heavy chain of anti-FLAG antibodies is indicated (*). These results are representative of 4 independent experiments, obtained with 2 different sources of Hrd1 vectors.


A)
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Cells
KCl (mM)
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kDa
75
50
37
**
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B)
Culture medium
Cells
MG132 (5 μM)
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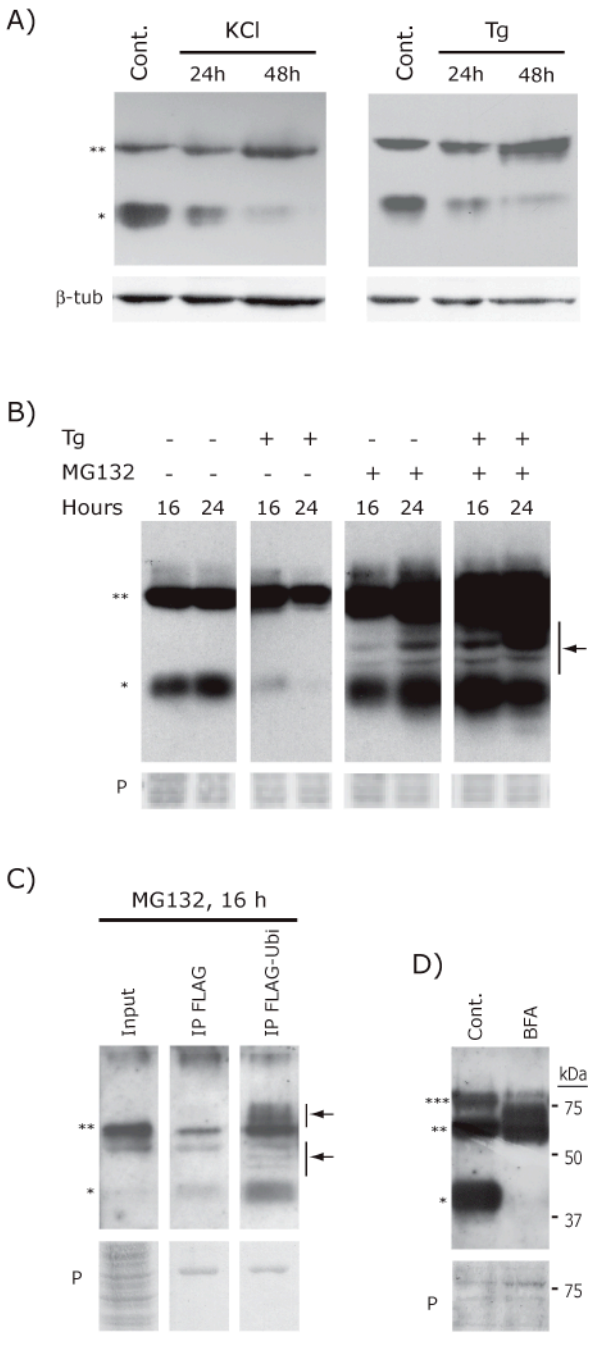
Figure 1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24
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C)
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Input
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Figure 2

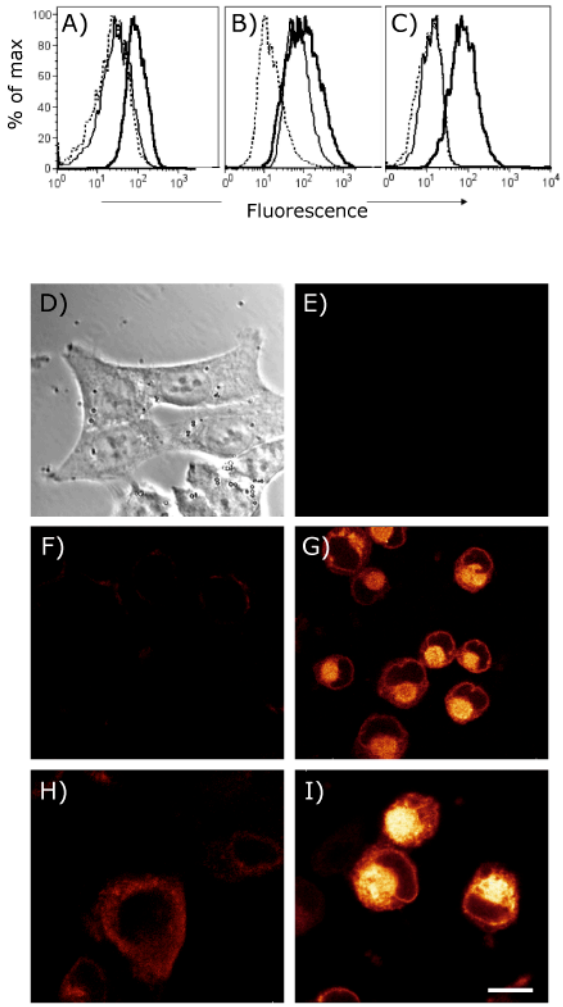
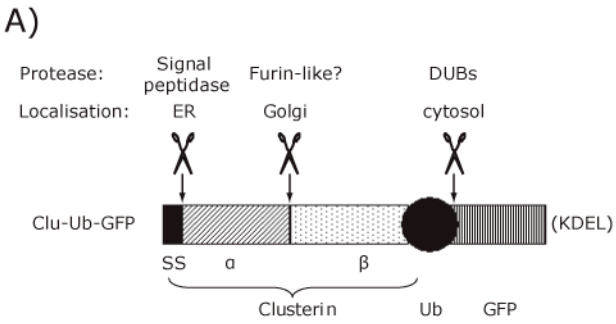


Figure 3


A)
Protease:
Signal peptidase
Furin-like?
DUBs
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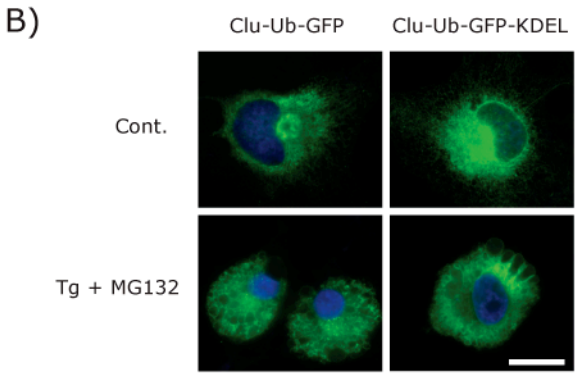
Golgi
cytosol
Clu-Ub-GFP
(KDEL)
SS
α
β
Clusterin
Ub
GFP


B)
Clu-Ub-GFP
Clu-Ub-GFP-KDEL
Cont.
Tg + MG132

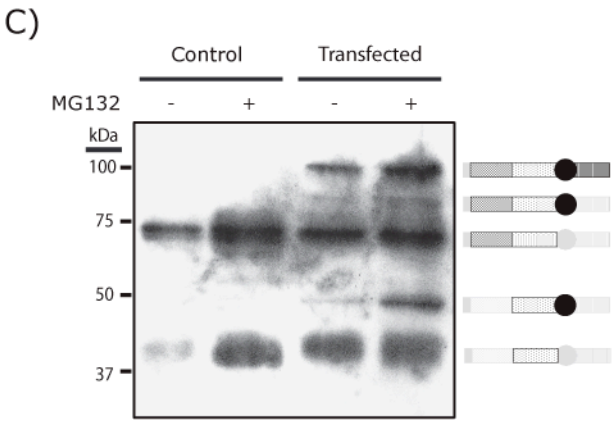

C)
Control
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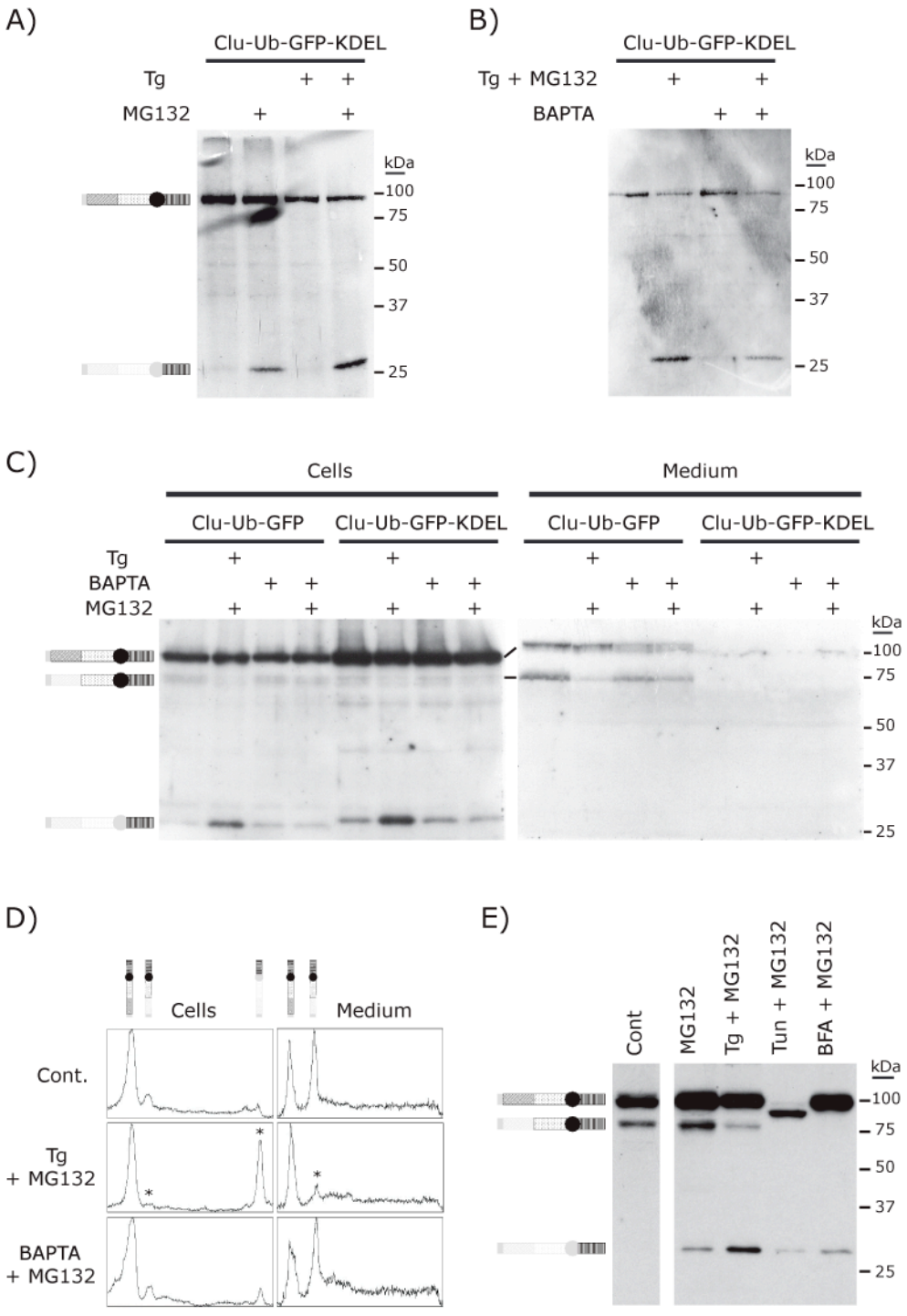
Figure 4


A)
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B)
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BAPTA
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37
25
C)
Cells
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Clu-Ub-GFP
Clu-Ub-GFP-KDEL
Clu-Ub-GFP
Clu-Ub-GFP-KDEL
Tg
BAPTA
MG132
kDa
100
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D)
Cells
Medium
Cont.
Tg
+ MG132
BAPTA
+ MG132
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Cont
MG132
Tg + MG132
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BFA + MG132
kDa
100
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25

Figure 5

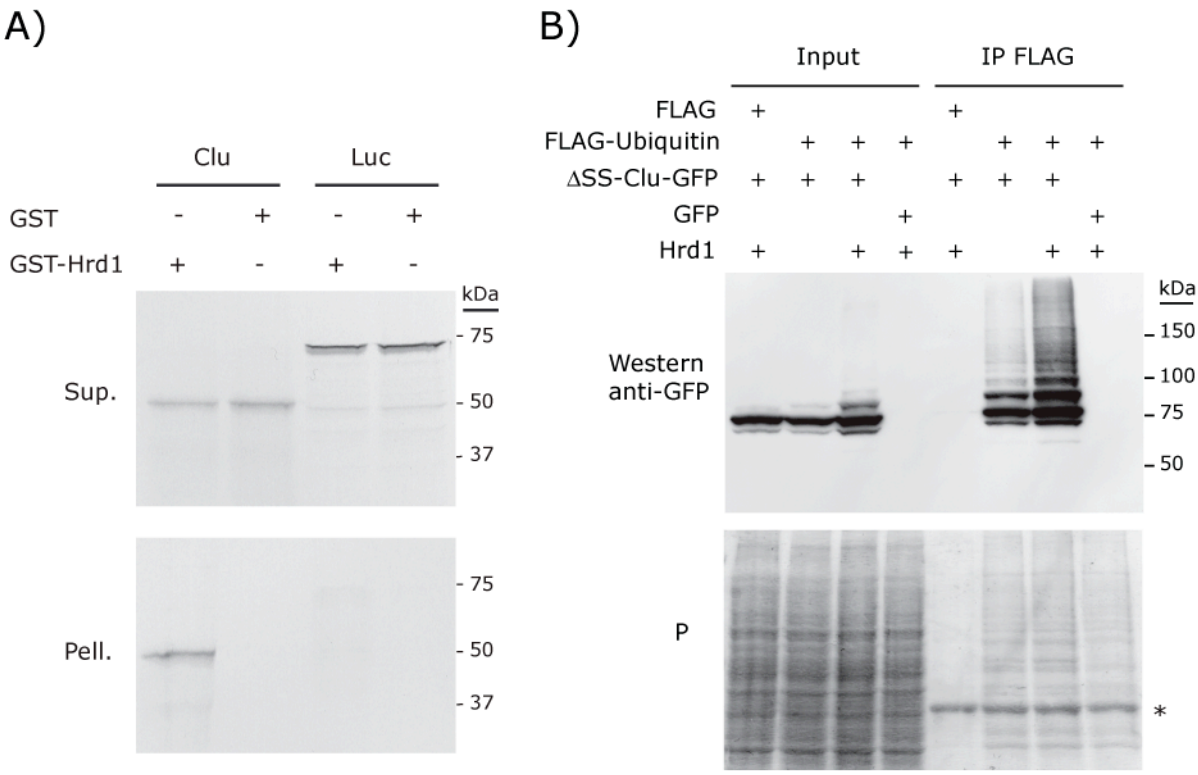
A)
Clu
Luc
GST
GST-Hrd1
kDa
Sup.
Pell.
75
50
37
B)
Input
IP FLAG
FLAG
FLAG-Ubiquitin
ΔSS-Clu-GFP
GFP
Hrd1
Western
anti-GFP
P
150
100
*

Figure 6