Intracellular clusterin causes juxtanuclear aggregate formation and mitochondrial alteration

Laure Deburse1,*, Jean-luc Vayssière2, Vincent Rincheval2, Fabien Loison1, Yves Le Dréan1 and Denis Michel1

1Information et Programmation Cellulaire, UMR6026 CNRS-Université de Rennes 1, Campus de Beaulieu, Bat. 13, 35042 Rennes Cedex, France
2CNRS-UPRES-A 8087, Laboratoire de génétique moléculaire et physiologique de l’EPHE, Université de Versailles/Saint-Quentin, Bâtiment Fermat, 45 avenue des Etats-Unis, 78035 Versailles Cedex, France

*Author for correspondence (e-mail: laure.deburse@univ-rennes1.fr)

Accepted 15 April 2003
Journal of Cell Science 116, 3109-3121 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00619

Summary
Clusterin is a puzzling protein upregulated in many diseased tissues, presented as either a survival or a death protein. The role of clusterin might depend on the final maturation and localization of the protein, which can be secreted or reside inside cells, either after in situ synthesis or uptake of extracellular clusterin. We studied the biological effects of intracellular clusterin and observed that clusterin forms containing the α-chain region strongly accumulated in an ubiquitinated form in juxtanuclear aggregates meeting the main criterions of aggresomes and leading to profound alterations of the mitochondrial network. The viability of cells transfected by intracellular forms of clusterin was improved by overexpression of Bcl-2, and caspase inhibition was capable of rescuing cells expressing clusterin, which presented an altered mitochondrial permeability. We propose that, although it might be an inherently pro-survival and anti-apoptotic protein expressed by cells under stress in an attempt to protect themselves, clusterin can become highly cytotoxic when accumulated in the intracellular compartment. This activity might reconcile the opposite purported influences of clusterin on cell survival and explain how clusterin can be causally involved in neurodegeneration.

Key words: Clusterin, Heat-shock protein, Aggresome, Mitochondria, Apoptosis, ΔΨm

Introduction
Clusterin has often been re-identified, under different names, as a gene overexpressed in situations of tissue regression, either developmentally programmed (Buttyan et al., 1989) or pathological (Jones and Jomary, 2002). Clusterin shares chaperone functions with heat-shock proteins (Hsps) (Humphreys et al., 1999), which would explain its capacity to interact with a wide range of partners (Wilson and Easterbrook-Smith, 2000) and with partially folded stressed proteins (DeMattos et al., 2002; Poon et al., 2002a). Accordingly, the clusterin gene transcription is responsive to cellular stress (Michel et al., 1997a). As for certain Hsps, clusterin expression was shown to confer resistance to apoptosis in some circumstances (Jomary et al., 1999; Sensibar et al., 1995). Conversely, a series of observations supported a link between clusterin and tissue involution. The clusterin gene expression has been shown induced in numerous cases of normally occurring apoptosis, such as the involution of temporary embryonic structures (Buttyan et al., 1989), the regression of hormone-dependent tissues such as the mammary gland (Guenette et al., 1994) or chicken ovarian atretic follicles (Mahon et al., 1999), and olfactory neurons after ablation of their synaptic target (Michel et al., 1994). Based on its sites of expression, clusterin is considered as a marker for tissue regression, specially in brain injuries (May and Finch, 1992). Clusterin accumulation has been reported in Alzheimer’s disease (May and Finch, 1992), epilepsy (Dragunow et al., 1995), excitotoxicity (Michel et al., 1992), scrapie infection (Duguid et al., 1989; Sasaki et al., 2002a), programmed neuronal degeneration (Norman et al., 1995) and normal ageing (Senut et al., 1992). However, the precise relationships between clusterin and apoptosis are still under debate. The coincidence between tissue involution and clusterin expression does not necessarily imply that clusterin is causally involved in apoptosis. For instance, contradictory results have been reported about the neuron loss induced by ischemia in clusterin-deficient mice (Han et al., 2001; Wehrli et al., 2001). This situation is further complicated by the complexity of clusterin localization and biogenesis pathways. Clusterin was formerly identified as a secreted protein. The clusterin precursor contains a typical secretion signal peptide and is matured as an heterodisulfide in the endoplasmic reticulum (ER), after proteolytic cleavage yielding the α and β subunits, and disulfide bond formation. Alternatively, clusterin can occur as a monomeric intracellular protein, in mammals (Reddy et al., 1996; Yang et al., 2001) and, to a higher extent, in chicken (Mahon et al., 1999). Besides, in prostatic cells undergoing apoptosis, clusterin could escape the secretory pathway and accumulate in the cytoplasmic compartment in a deglycosylated form (Lakins et al., 1998). Finally, clusterin could accumulate in cells that do not transcribe the clusterin gene, possibly through an uptake of extracellular clusterin, particularly by neurons (Pasinetti et al., 1994). This last situation has been reported in the injured nervous system, in which neurons can internalize extracellular clusterin secreted by glial cells in response to neuronal insults.
 Rat clusterin cDNA deleted from its secretion signal coding sequence

The monomeric full-length clusterin was produced from a truncated DNA constructs

Probes and ZV AD (Z-Val-Ala-DL-Asp-fluoromethylketone) (final concentration 50 nM or 100 nM) from Molecular

used. Nocodazole (final concentration 10

antibody (sc-2020, Santa Cruz Biotechnology, 1:5000) and enhanced donkey anti-goat horseradish-peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories, 1:50). For western blotting, red-X-conjugated secondary antibodies (715-295-150, 711-295-152, Slide)-clu encoding vectors mixed with LipofectAMINE PLUS™ reagent (Gibco-BRL) according to manufacturer’s recommendations. Flow cytometry analyses were performed 24 hours after transfection. When tested, ZVAD was added at the same time as transfection DNA.

Cell culture and transfection

COS-7 cells were grown (15,000 cells cm−2) at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies) in 35 mm dishes for fluorescence microscopy and 82 mm dishes for immunoblotting experiences. At 70% of confluence, the cells were transiently transfected using a calcium phosphate co-precipitation method with 4 μg of DNA for fluorescence microscopy and 17 μg total DNA for immunoblotting. When necessary, nocodazole or MG-132 were added 36 hours after transfection for 6 or 12 hours, respectively. HeLa/Bcl-2 cells were plated in 35 mm dishes at 70% of confluence, the cells were transfected with 1 μg total DNA per dish of either GFP, GFP-ΔSP-clu, GFP-α-clu or GFP-β-clu encoding vectors mixed with LipofectAMINE PLUS™ reagent (Gibco-BRL) according to manufacturer’s recommendations. Flow cytometry analyses were performed 24 hours after transfection. When tested, ZVAD was added at the same time as transfection DNA.

Immunocytochemistry

COS-7 cells were grown on poly-D-lysine coated glass coverslips and transiently transfected with 4 μg of pEGFP-α-clu or pEGFP-ΔSP-clu. After 36 hours, cells were rinsed in PBS and fixed in −20°C methanol for 6 minutes. The plated cells were washed in PBS and incubated in permeabilizing and blocking buffer (0.2% Triton X-100 and 5% non-fat milk in PBS) at room temperature for 2 hours. Immunostaining was performed by incubation at 4°C overnight with the appropriate primary antibody. Coverslips were washed, incubated for 2 hours with secondary antibody, washed in PBS, counterstained with DAPI and mounted on slides with Mowiol.

Fluorescence microscopy

Immunofluorescence microscopy analysis was performed using an Olympus AX70 and AnalySIS software with the following Olympus filters: MWB for GFP (excitation at 450-480 nm, emission above 515 nm); MWIY for rhodamine red X (excitation at 545-580 nm, emission above 610 nm); and MWU for DAPI (excitation at 330-385 nm, emission above 420 nm). Mitochondrial localization was assessed with the mitochondrion-selective dye MitoTracker Red™. Incubation at 37°C for 30 minutes (100 nM) allowed its accumulation in mitochondria of living cells. Observation was performed immediately after washing with fresh medium under an Olympus IX70 microscope. Filters MWB and MWG (excitation at 510-550 nm, emission above...
Cell extracts were prepared by freeze-thaw lysis in 20 mM HEPES of pEGFP-DYA cell-permeable marker that, at low doses (50 nM), specifically
enhanced chemiluminescence.

Immunoblotting

COS-7 cells were transiently transfected with 3 µg pCMV-β-gal, 7 µg of pEGFP-ΔSP-clu and 7 µg of pCMV-FLAG-Ubi or pCMV-FLAG-K48R-Ubi or empty vector pCMV-FLAG. If necessary, after 36 hours, 10 µM of MG-132 was added for 6 hours before protein extraction. Cell extracts were prepared by freeze-thaw lysis in 20 mM HEPES (N-2-hydroxethylpiperazine-N’-2-ethanesulfonic acid, pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol and protease inhibitor cocktail (Complete™, Roche). The soluble and pellet fractions were obtained by ultracentrifugation at 100,000 g for 5 minutes in a Beckman TL100. For immunoblotting, cell extracts were normalized with β-galactosidase activities. The reporter gene (in pCMV-β-gal) was co-transfected with GFP-chimera and ubiquitin expression plasmids to be used as a control for transfection efficiency. 10% of soluble cell extracts were incubated at 37°C in 0.1 M sodium phosphate buffer, pH 7.5, 1.25 mM MgCl₂, 56 mM 2-mercaptoethanol, 1.1 mg ml⁻¹ 2-nitrophenyl-β-D-galactopyranoside. β-Galactosidase activities were measured by optical density at 405 nm. Supernatants and pellets were solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 8% glycerol and 5% 2-mercaptoethanol) and resolved by 10% SDS-PAGE and transferred to nitrocellulose. Clusterin was detected using a goat polyclonal antibody, a horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Flow cytometry analyses

Mitochondrial membrane potential was assessed by the retention of Mitotracker Red (CMX-Ros). This cationic lipophilic fluorochrome is a cell-permeable marker that, at low doses (50 nM), specifically accumulates in mitochondria in mammalian cells in proportion to ΔΨm (Poot et al., 1996). Cell staining were performed as follows. Cells were harvested 24 hours after transfection, centrifuged and resuspended in complete medium at a concentration of 10⁶ cells ml⁻¹. Cells were then loaded with 50 nM Mitotracker Red for 30 minutes at 37°C in an humidified 5% CO₂/95% air incubator. Flow cytometry measurements were performed on an XL3C flow cytometer (Beckman-Coulter France). Fluorescence excitation was obtained using the blue wavelength (488 nm) of an argon ion laser operating at 15 mW. Green fluorescence of GFP was collected with a 525 nm band pass filter and red fluorescence of Mitotracker Red with a 620 nm band-pass filter. Analyses were performed on 10⁴ cells and data were stored in list mode. Light-scattering values were measured on a linear scale of 1024 channels and fluorescence intensities on a logarithmic scale of fluorescence.

Results

Localization of the various intracellular forms of clusterin

Because the complex molecular mechanisms underlying the synthesis of the different forms of clusterin in vivo cannot be controlled experimentally, we directed the synthesis of GFP fusions with intracellular clusterin. We constructed a transgene named GFP-ΔSP-clu (Fig. 1A), mimicking the full-length monomeric clusterin produced in situ. Because an important aim is to determine the subsequent fate of clusterin internalized by cells and probably leading to neuron death, we also made constructs allowing the synthesis of GFP fusions with α or β chains, corresponding to the isolated subunits of secreted heterodisulfide clusterin (GFP-α-clu and GFP-β-clu, Fig. 1A). Proteins derived from these constructs exhibited different but reproducible subcellular patterns (Fig. 1B). GFP-ΔSP-clu fluorescence was mainly diffuse in the cytosol, with a juxtanuclear aggregate. GFP-α-clu appeared mostly aggregated in a cellular background completely devoid of soluble fluorescence. The size and form of aggregates were dependent on the efficiency of transfection and on the time after transfection, ranging from a juxtanuclear dot (Fig. 1B) to massive crescent- or ring-shaped perinuclear aggregates (Fig. 1D). GFP-β-clu was almost exclusively diffused except for a faint perinuclear ring (Fig. 1B), suggesting the existence of some nuclear targeting signal, and rare cells presenting a small juxtanuclear aggregate (not shown). We then wanted to check whether the propensity to aggregate of GFP-ΔSP-clu and GFP-α-clu could be an artefact caused by the GFP fusion. Indeed, GFP was shown to profoundly modify the solubility of fusion proteins in an unpredictable manner when fused to either the N- or C-terminus of a protein (Garcia-Mata et al., 1999). To test such a possibility for clusterin, we examined by immunocytochemistry the distribution of α-subunit and full-length clusterin devoid of any fusion protein. As shown in Fig. 1C, their distributions were similar to those of GFP-fused chimeras, either in small or large juxtanuclear deposits. Interestingly, advanced stages of GFP-α-clu aggregation yielded images with massive perinuclear aggregates that were very reminiscent of those obtained after clusterin immunostaining in neurons from senescent rat brain and described as punctate cytoplasmic labeling and intensely stained granules with no visible cell membrane (Senut et al., 1992) (Fig. 1D).

Structural characterization of juxtanuclear α-clu deposits as aggresomes

We checked clusterin aggregates for the cytological criterions of the recently described structures known as aggresomes. Aggresomes are perinuclear inclusions forming specifically at the centrosome and ensheathed in membrane-free vimentin cages, and were initially described as misfolded, ubiquitinated protein inclusions (Johnston et al., 1998). Aggresomes were also shown to recruit proteasome subunits as well as Hsps, and are believed to be involved in the balance between protein folding, aggregation and degradation (Fabburni et al., 2000). COS-7 cells were transfected with GFP-α-clu and fixed to proceed to immunochemistry against the markers of aggresomes. Immunostaining against γ-tubulin, a component of the centrosome and of the pericentriolar material, showed two discrete dots corresponding to centrioles, recognizable in mitotic cells (Fig. 2, arrowhead shows a centriole during metaphase). GFP-α-clu deposits were detected at the centrosome location and a strong γ-tubulin immunostaining was observed within the aggregates. Juxtanuclear inclusions of GFP-ΔSP-clu showed the same location at the centrosome (data not shown). In cells overexpressing GFP-α-clu, a network of vimentin collapsed and concentrated at the level of juxtanuclear deposits, in contrast to untransfected control cells from the same dishes, in which vimentin immunostained filaments formed a network extending from a juxtanuclear focus (the intermediate filament organization center) to the periphery (Fig. 2). Immunostaining of the MSS1 subunit of the 19S proteasomal complex revealed a dotted distribution in control cells but a strong enrichment at the level of GFP-α-clu.
aggregates in transfected cells (Fig. 2). Finally, we tested the localization of the Hsp70 protein chaperone. Hsp70 immunostaining was mainly homogeneous and cytoplasmic in untransfected cells but, in transfected cells, it was concentrated at the level of GFP-α-clu inclusions (Fig. 2). These co-localizations were systematically observed in all cells expressing constructs, in at least two distinct experiments. Together, these co-localization experiments allowed us to classify GFP-α-clu inclusions as aggresomes.

Intracellular GFP-α-clu aggregates share functional features with aggresomes

In addition to the presence of the molecular markers listed above, aggresomes also have functional characteristics. Aggresome formation strictly requires functional microtubules (Kopito, 2000) and can be antagonized by the protein chaperone Hsp70 (Dul et al., 2001). We tested the effect of the microtubule-disrupting agent nocodazole on GFP-α-clu aggregation. Incubation with nocodazole dramatically prevented the formation of juxtanuclear clusterin aggregates and stabilized peripheral cytoplasmic fluorescent dots (Fig. 3C,D). Treatment with nocodazole reduced from 75% to 25% (n=200) the proportion of cells presenting one large juxtanuclear aggregate. This result is a strong indication that clusterin deposits form at the microtubule organizing center (MTOC) by pericentriolar accretion of peripheral small protein aggregates. Aggresome formation is also believed to be initiated by misfolded proteins, abnormally exposing buried domains that are hydrophobic and pro-aggregative. The protein chaperone Hsp70, binding with high affinity to such domains, has frequently been shown to be capable of preventing or reversing protein precipitation processes. We tested this possibility in the case of the GFP-α-clu aggresomes. As shown in Fig. 3E,F, co-transfection of COS-7 cells with a fourfold excess of Hsp70 expression vector relative to GFP-α-clu led to a striking reduction in the size of aggresomes, in parallel with an increase in the diffuse fluorescence background. Hsp70 co-transfection increased the number of GFP-α-clu-positive cells presenting soluble GFP fluorescence from 30% to 80% (n=200). Similar effects of Hsp70 and nocodazole have been observed on the smaller GFP-ΔSP-clu juxtanuclear aggregates (data not shown).

Ubiquitination of intracellular clusterin

Because aggresomes frequently immunostain for ubiquitin, we tested for the presence of ubiquitin in clusterin inclusions. Transfection of COS-7 cells with the two clusterin constructs forming aggregates led to a redistribution of the cellular
Clusterin aggresomes

ubiquitin content (Fig. 4A), with a strong and reproducible concentration at the level of clusterin aggregates. Such a co-localization of ubiquitin into aggresomes does not necessarily imply that aggregated proteins are ubiquitinated. Considering the striking superposition of ubiquitin immunoreactivity and GFP-clusterin fluorescence, we wanted to check whether this co-localization was the consequence of a mere accumulation of ubiquitin into aggresomes or instead of a covalent ligation of ubiquitin to clusterin. For this purpose, we co-transfected COS-7 cells with GFP-ΔSP-clu and plasmids expressing ubiquitin, either normal or carrying the K48R amino acid substitution. This mutation acts as a K48-G76 polyubiquitin chain terminator, leading to an enrichment in monoubiquitinated forms (Chau et al., 1989). Transfected cells were treated or not with the proteasome inhibitor MG-132 before western blot clusterin immunodetection. In conditions of wild-type ubiquitin overexpression and MG-132 treatment, clusterin immunostaining revealed a series of high molecular weight bands in both supernatant and pellet fractions (Fig. 4B). These bands are likely to correspond to polyubiquitinated clusterin, because they accumulated upon proteasome inhibition and were no longer visible when using mutant ubiquitin, except for the smallest one, which probably corresponds to monoubiquitinated clusterin. In control conditions, in which only empty vector was used, high

Fig. 2. GFP-α-clu juxtanuclear inclusions are immunoreactive for different aggresome markers. 36 hours after transfection with GFP-α-clu, COS-7 cells were fixed, permeabilized and incubated with antibodies against γ-tubulin, vimentin, the MSS1 subunit of 19S complex of proteasome and Hsp70, coupled with rhodamine-conjugated secondary antibodies. Nucleus was counterstained with DAPI. For all of these markers, intense immunostaining co-localized with the clusterin aggresome in a very reproducible manner. Arrowhead indicates centriole position after γ-tubulin labeling. Bar, 10 μm.

Fig. 3. GFP-α-clu aggregates share functional features with aggresomes. Perinuclear relocalization of peripheral GFP-α-clu aggregates can be prevented by nocodazole, a microtubule-disrupting agent and by the chaperone Hsp70. Addition of 10 μg ml⁻¹ nocodazole during 12 hours in culture medium led to the scattering of GFP-α-clu into multiple dots (C,D), without formation of massive juxtanuclear aggregates observed in controls (A,B). COS-7 cells overexpressing GFP-α-clu and Hsp70 in a fourfold excess presented a strong size reduction of juxtanuclear aggregates and an increase of the diffuse cytoplasmic distribution (E,F). Photographs are highly representative of the effect of the treatments observed in a large population of cells. Bar, 10 μm.
molecular weight bands were also observed in presence of proteasome inhibitor but only in the insoluble fraction corresponding to aggresomes. Thus, under these conditions, ubiquitination of clusterin seems to favor its precipitation. Together, these observations strongly suggest that intracellular clusterin is a major substrate for polyubiquitination and that aggresomes are enriched in multiubiquitinated clusterin.

Alteration of the mitochondrial distribution upon clusterin aggresome formation
Permanent aggresomes have been shown to compromise cell viability but the cellular and molecular basis of their toxicity remains puzzling. We observed that clusterin aggresome formation led to dramatic mitochondrial changes. We first compared the distribution of the mitochondrial dye MitoTracker Red between untransfected and GFP-α-CLU-overexpressing cells. The top panels of Fig. 5A show that MitoTracker Red was strongly concentrated at the level of clusterin aggresomes and that the cytoplasmic mitochondrial density was lowered (compare transfected and non-transfected cells). Because the localization of artificial dyes such as MitoTracker Red is not fully specific and might be influenced by the presence of protein aggregates, we wanted to verify the relocation of mitochondria through an immunological detection of a mitochondrial protein. Immunostaining of COX6C, the cytochrome c oxidase subunit VIc, yielded images perfectly similar to those obtained with MitoTracker Red (Fig. 5A). However, the close overlap between mitochondrial markers and clusterin aggresomes did not reflect a precise localization of clusterin at the level of mitochondria. Fig. 5B clearly shows that clusterin and mitochondria co-localization depended on the degree of aggregation of clusterin. In cells still presenting scattered aggregates, the most peripheral clusterin and mitochondrial signals were clearly dissociated except in densely stained areas. Hence, GFP-α-CLU aggresome formation led to a strong concentration of mitochondria and to a decrease of the peripheral mitochondrial density. These changes were also observed for GFP-ΔSP-CLU aggregates but not for GFP-β-CLU, which did not form aggresomes (data not shown).

Intracellular clusterin fragments trigger a mitochondria-mediated apoptotic pathway
Transfection of cells with clusterin constructs often led to the appearance of apoptotic nuclei and of floating fluorescent cells with highly condensed cytoplasm, reminiscent of apoptotic cells. Although these figures were transient and rapidly lost their GFP fluorescence, they could be detected by fluorimetric analysis. As shown in Fig. 6, more small fluorescent bodies were detected after transfection with GFP-clusterin fusions compared with GFP alone (Fig. 6A, compare left panels for small sizes and right panels for large sizes between all constructs; see Fig. 6B for mean values). We wanted to evaluate the possible induction of mitochondrion-mediated apoptosis in cells containing clusterin aggregates by examining the effects of Bcl-2 overexpression and caspase inhibition. For this purpose, we have developed a stable HeLa cell line overexpressing Bcl-2 in a tetracycline-regulated manner. In the presence of tetracycline, expression of Bcl-2 is inhibited and removal of tetracycline allows Bcl-2 overexpression. Cells overexpressing or not Bcl-2 were transfected with the different
Clusterin aggresomes

GFP-clusterin constructs. We first observed that the proportion of small fluorescent cells was strikingly lowered upon Bcl-2 induction in cell populations transfected with GFP-clusterin constructs compared with those transfected with GFP alone (Fig. 6A, compare left and right panels; see Fig. 6B for mean values).

The protective effect of Bcl-2 suggested that mitochondrial defect could be involved in the triggering of the apoptotic program in cells containing clusterin. It has been shown that the mitochondrial membrane potential ($\Delta \Psi_m^m$) drop can be an early apoptosis event in promoting mitochondrial outer membrane permeabilization and activation of the mitochondrial apoptotic pathway (Desagher and Martinou, 2000; Vayssière et al., 1994). Therefore, we asked whether GFP-clusterin indeed provokes $\Delta \Psi_m^m$ collapse by the mean of flow cytometry analyses. We assessed mitochondrial membrane potential using MitoTracker Red, a lipophilic cation taken up by mitochondria in proportion to $\Delta \Psi_m^m$ (Macho et al., 1996). This study was performed on GFP-clusterin-transfected cells overexpressing or not Bcl-2 and, in the latter case, in the presence or not of ZV AD, a broad-spectrum caspase inhibitor. For each sample, mitochondrial membrane potential and cell size, as respectively measured by MitoTracker Red fluorescence and forward scatter value (FSC) and Fig. 7B for mean values) compared with GFP-only expression (9%). Simultaneous overexpression of Bcl-2 greatly reduced the percentage of cells presenting a $\Delta \Psi_m^m$ drop, to 15%, 8% and 9% for GFP-ASP-clu, GFP-α-clu, and GFP-β-clu, respectively (Fig. 7Ab,e,h and Fig 7B). ZV AD addition, by contrast, induced a significant increase of dying cells, from 28% to 64% for GFP-ASP-clu, from 23% to 62% for GFP-α-clu and from 27% to 58% for GFP-β-clu, but it had only minor effects on the proportion of dying GFP-expressing cells (Fig. 7Ac,f,i,l and Fig. 7B). Taking into account the effects of Bcl-2 and ZV AD on the viability of cells, it can be proposed that Bcl-2 exerts its protective role by preventing commitment of GFP-ASP-clu, GFP-α-clu or GFP-β-clu expressing cells to apoptosis, whereas ZV AD-mediated caspase inhibition rescues early apoptotic cells with lower $\Delta \Psi_m^m$ in delaying completion of the apoptotic program (McCarthy et al., 1997). These data suggest that intracellular clusterin can trigger the mitochondrial signaling pathway of apoptosis.

**Discussion**

Hsp and chaperones are believed to be cytoprotective proteins capable of solubilizing unfolded or aggregated proteins. They

---

**Fig. 5.** Formation of clusterin aggresome induced relocation of mitochondria.
(A) COS-7 cells transfected with GFP-α-clu were incubated for 15 minutes with 100 nM MitoTracker Red™, fixed, counterstained with DAPI and then analysed by fluorescence microscopy. In cells presenting clusterin aggresomes, mitochondria collapsed and massively relocated at the juxtanuclear aggresome. The same results were obtained with immunostaining using a COX6C antibody coupled with rhodamine-conjugated secondary antibody. Bar, 10 μm for MitoTracker Red and COX6C labeling. (B) The co-localization of mitochondria and GFP-α-clu is visible only at the level of large GFP-α-clu aggregates. Bar, 10 μm.
recognize hydrophobic surfaces of denatured proteins and keep them in solution instead of forming insoluble and potentially damaging precipitates. Hsps are also endowed with highly hydrophobic domains that can bind those of denatured proteins, but that are also aggregation-prone in certain circumstances. The paradoxical propensity of certain Hsps to enhance protein inclusions has already been reported for several Hsps. For example, mammalian α-B-crystallins clearly tend to form aggregates in certain neurological pathologies (Iwaki et al., 1989; Tamaoka et al., 1995). Hsp104 can enhance the formation of proteins inclusions in yeast (Newnam et al., 1999) and Hsp40 can increase protein aggregate formation in mammalian cells (Wytenbach et al., 2000). Our work suggests that clusterin, which has also been proposed to be a protein chaperone largely composed of sticky amphipathic α-helices and membrane-interacting and aggregation-prone domains (Bailey et al., 2001), can also enhance aggresome formation when accumulating inside cells.

**Aggresome-forming activity of clusterin**

We showed that α-chain of clusterin has a strong aggresome-forming capacity compared with previously described aggresome-forming proteins, and that this activity was obtained in the absence of proteasome inhibition. Under the same conditions, full-length clusterin (which exhibited a cytosolic, soluble distribution) also formed smaller juxtanuclear inclusions. Aggresomes are recently defined structures, primarily identified as sites of accumulation of misfolded proteins such as the mutated cystic fibrosis conductance regulator (CFTR) (Johnston et al., 1998). The established functional and structural features of aggresomes were reproduced for GFP-α-clu aggregates, such as the formation at centrosomes by accretion of peripheral aggregates via microtubular tracts, and the co-localization of components of protein quality control systems: proteasome subunits, ubiquitin and protein chaperones. The relationships between nuclear or cytoplasmic protein aggregation structures and pathologies remain obscure. Although it was first associated with protein diseases, aggresome formation has since been interpreted as a mechanism of protection against the deleterious spreading of misfolded proteins in the cell (Chung et al., 2001). However, certain proteins appear to resist to these degradation factories, such as polyglutamine-rich or misfolded proteins with exposed hydrophobic aggregation-prone domains. These resistant aggresomes can cause secondary cellular troubles, leading to cell death. The present work suggests that this can be the case for clusterin accumulated in diseased tissues and whose expression is exacerbated in situations of tissue stresses. The physiological existence of isolated clusterin α-chain has not been demonstrated. It could correspond to fragment dissociated after uptake of secreted clusterin in the reducing intracellular compartment in presence of glutathione, but data from Humphreys et al. (Humphreys et
**Clusterin aggresomes**

3117

**Fig. 7.** Effects of Bcl-2 and ZVAD on mitochondrial events associated with clusterin-induced apoptosis of HeLa cells by multiparametric analysis of HeLa/Bcl-2 cells by flow cytometry. Each cell is represented by a dot. The data are relative to GFP-ΔSP-clu-, GFP-α-clu-, GFP-β-clu- and GFP-transfected cells, overexpressing (b,e,h,k) or not (a,c,d,f,g,i,j,l) Bcl-2 and in the presence (c,f,i,l) or not (a,b,d,e,g,h,j,k) of ZVAD. For each sample, the mitochondrial membrane potential as measured by MitoTracker Red™ fluorescence was reported in cyogram (MitoTracker Red versus size). Only GFP-positive cells with normal size were considered in multiparametric cyograms. Cells can be separated into two populations based on their MitoTracker Red fluorescence: one with high fluorescence (top) and the other with lower fluorescence (bottom). The proportion of cells in each population is specified in each panel of the cyograms (percentages correspond to only one experiment). The expression of GFP-clusterin constructs induced a loss of mitochondrial membrane potential, but cell size was not affected. (B) Proportion of variation of normally sized GFP-positive and MitoTracker-negative cells after Bcl-2 induction (black bars), ZVAD treatment (gray bars) or no Bcl-2 induction and no caspase inhibition (white bars). Values were calculated from three independent transfection experiments. Each bar represents the mean value±s.d. (n=3). *P<0.05 significantly different from control values; NS, not significantly different in Student’s t-test.

...be surprising, because hydrophobic domains have been described all along the clusterin precursor (Bailey et al., 2001). These results suggest that the various intracellular clusterin fragments proposed in the literature, either full-length or truncated and carrying the α region, should have comparable effects when accumulating inside cells. Indeed, monomeric full-length or truncated clusterin fragments seem capable of accumulating intracellularly after in situ synthesis, sometimes without passing through the ER-to-Golgi traffic. Such situations have been reported in chicken (Mahon et al., 1999) and mammals (Lakins et al., 1998; Mahon et al., 1999; Reddy et al., 1996; Yang et al., 2000).

Clusterin aggregation and the ubiquitin-proteasome system

The way by which intracellular protein aggregation is deleterious for cells remains elusive. Some hypotheses have been proposed, such as a dysregulation of the ubiquitin-proteasome pathway. If unfolded proteins escape degradation, 19S particles of proteasomes are supposed to be sequestered by the growing protein aggregates, resulting in a depletion of proteasomal activity and cellular dysfunction. This possibility cannot be ruled out for clusterin aggresomes because we showed that they are also enriched in proteasome subunits. Another classical feature of aggresomes is the accumulation of ubiquitin, suggesting that they contain...
misfolded or abnormally folded proteins potentially targeted for but resistant to proteasomal degradation. Certain aggresome-forming proteins, such as the mutant CFTR, have been shown to be polyubiquitinated in aggresomes (Johnston et al., 1998) but this has not yet been demonstrated in vivo for polyglutamine-containing proteins (Sakahira et al., 2002). We showed that ubiquitin strongly concentrated only in transfected cells containing clusterin aggregates. In addition, our biochemical studies strongly suggest that clusterin is a major ubiquitination substrate. The biological significance of the intense polyubiquitination of certain aggregated proteins is currently unclear. It can reflect the inability of proteasome to degrade these ubiquitinated substrates or the aggregation propensity of polyubiquitinated proteins (Cyr et al., 2002). Our results are in line with this last hypothesis because overexpression of a mutant ubiquitin acting as a K48-G76 polyubiquitin chain terminator inhibited apparent clusterin polyubiquitination. Alternatively, there is the attractive possibility that protein modification with monoubiquitin or short polyubiquitin chains can be a proteasome-independent post-translational modification of the protein surface avoiding extended aggregation (Gray, 2001). However, this hypothesis is not supported for clusterin by our experiment using the K48R mutant ubiquitin. This polyubiquitin-chain-terminating molecule abolished the formation of high molecular weight conjugated clusterin, which would not be the case if these bands corresponded to clusterin monoubiquitinated at the level of different lysines. We showed that overexpression of Hsp70 can prevent or decrease aggresome formation by GFP-α-clu. This feature, reported for many slowly aggregating disease proteins, suggests that aggregation of clusterin proceeds through an intermediate unfolded state with exposed hydrophobic interfaces. This view is supported by the existence of amphipathic α-helices and molten globule-like regions in clusterin (Bailey et al., 2001).

Alteration of mitochondrial integrity by intracellular clusterin

Intracellular clusterin has already shown long-term toxicity in stable expression experiments (Yang et al., 2000). We also failed to obtain cell lines stably expressing intracellular clusterin, unlike secreted clusterin (data not shown). We show here that clusterin can initiate a mitochondrion-dependent apoptosis program. Apoptosis is a mode of cell death used by multicellular organism to eradicate cells in diverse physiological and pathological settings (Kaufmann and Hengartner, 2001). Apoptotic cell demolition is orchestrated by a family of cysteine proteases, the caspases, which act in cascade. One major caspase activation cascade is triggered by cytochrome c release from the intermembrane space of mitochondria. In the cytosol, cytochrome c binds Apaf-1 forming an oligomeric complex (apoptosome), which recruits procaspase-9 and induces its autoactivation. Caspase-9 in turn activates downstream caspases including caspase-3. Proteins of the Bcl-2 family are major regulators of this mitochondrial pathway. Notably, the antiapoptotic Bcl-2 protein inhibits apoptosis by preserving mitochondrial integrity. The intracellular accumulation of clusterin fragments clearly triggered an alteration of mitochondrial parameters, because Bcl-2 overexpression strongly improved the viability of cells expressing clusterin. Moreover, clusterin clearly led to mitochondrial changes associated with a decrease in ΔΨm. In the mitochondrion-associated apoptosis cascade, this parameter is considered as an event upstream of caspase activation. The fact that caspase inhibition can rescue clusterin-positive cells with low ΔΨm from cell death is consistent with a causal relationship between intracellular clusterin accumulation and caspase activation, although this remains to be established.

We also observed that cells containing GFP-α-clu aggresomes present dramatic perinuclear recanalizations of mitochondria. Mitochondrial markers, such as the dye MitoTracker Red and the enzyme COX6C, shifted from a typical scattered pattern to an intense staining at the level of condensed aggresomes. Such mitochondrial clustering had already been observed in cells overexpressing the pro-apoptotic proteins Bax (Desagher and Martinou, 2000) and t-Bid (Li et al., 1998), and in cells containing aggresomes driven by mutant huntingtin fragments (Waelter et al., 2001). Several hypotheses about the molecular bases of aggresome toxicity have been proposed, such as sequestration of essential regulatory proteins involved in key cellular events, such as transcription factors or proteasomes. These observations also suggest the possibility of a mitochondrial network breakdown. However, the results obtained with GFP-β-clu, which does not form aggresomes, indicate that the mitochondrion-associated apoptosis caused by clusterin is not necessarily associated with the redistribution of mitochondria. Two hypotheses can be raised to explain this observation. On the one hand, the disruption of mitochondrial network caused by clusterin aggresomes could be irrelevant to the effect on ΔΨm. The effect of the relocation of these organelles at the centrosome on cellular physiology should be elucidated. On the other hand, mitochondrial aggregation could be causally related to the decrease of mitochondrial potential and another mechanism could be postulated to explain the β-chain toxicity. Considering that the C-terminal portion of clusterin has been shown to be capable of inducing cell death through an unknown mechanism involving the KU70 nuclear protein (Yang et al., 2000), it is possible that this toxicity could also be mediated by mitochondrion-dependent apoptosis.

Biological importance of intracellular clusterin accumulation

Considering the present results, the elucidation of the molecular mechanisms underlying the intracellular accumulation of clusterin is of great importance. One possibility suggested by the literature is the direct synthesis in situ of cytosolic forms of clusterin (Lakins et al., 1998; Mahon et al., 1999; Reddy et al., 1996; Yang et al., 2000). Another possibility would be an escape from the ER and Golgi apparatus of clusterin forms destined to secretion. Disruption of the ER and Golgi has been shown for several misfolded mutant protein (Graves et al., 2001; Stieber et al., 2000), which can escape the ER, exceed the proteasomal system and accumulate in the cytoplasm (Kopito, 2000). Such a phenomenon could occur for clusterin, which is synthesized in large amounts in insulted tissues and is an intrinsically disordered protein (Bailey et al., 2001). This possibility is also
in line with the potent aggregation propensity of clusterin revealed in this study, and could explain the intracellular accumulation and conformational changes of clusterin observed in clusterin-synthesizing cells dying in the involving prostate (Lakins et al., 1998).

Alternatively, the cellular uptake of extracellular clusterin has repeatedly been suggested, particularly for neurons (Han et al., 2001; Michel et al., 1997b; Pasinetti et al., 1994; Walton et al., 1996), but the precise mode of clusterin internalization is not known. Several endocytosis receptors for clusterin have been proposed (Mahon et al., 1999), including Megalin/gp330, which is also a receptor for ApoE (Kounnas et al., 1995) [a protein upregulated in neurons destined to die in the Alzheimer’s disease (LaFerla et al., 1997)]. After endocytic internalization, clusterin could escape the endosomal compartment to enter the cytosol and produce its biological effect. The intracellular trafficking from endosomes to the cytosol has been clearly reported for certain bacterial toxins, such as diphtheria toxin (Falnes and Sandvig, 2000). This toxin is composed of two subunits linked by disulfide bonds that are derived from a single protein precursor after proteolytic cleavage. After endocytosis, the acidic pH of early endosomes leads to the exposure of the lipid-interacting domains of diphtheria toxin and then to its escape in the cytoplasm. Interestingly, low pH has also been shown to increase the exposure of hydrophobic regions on clusterin (Poon et al., 2002b). This behavior is strikingly similar to that of diphtheria toxin, suggesting the possibility of an escape of clusterin from endosomes to cytoplasm.

After endocytosis, other toxins undergo retrograde transport to reach the ER lumen, from where they translocate into the cytosol (Falnes and Sandvig, 2000). These toxins probably use the ER-associated protein degradation pathway for this intracellular transport (Simpson et al., 1999), like unfolded secretory or membrane proteins also known to be moved from the ER to the cytoplasm for degradation by the ubiquitin-proteasome system (Kopito, 2000).

Finally, though clusterin appears to be mainly intracellular in chicken, in other species, it has been described as an atypical Hsp in that it is secreted outside synthesizing cells (Poon et al., 2000). In fact, its capacity for secretion seems to be also a general feature of classical Hsps, which has also been shown to be released from cells (Multhoff and Hightower, 1996). Moreover, some molecular chaperones were found capable of cell-cell trafficking from glia to neurons (Tytell et al., 1986) through an unconventional mechanism that allows their entry into the cytosol (Fujihara and Nadler, 1999). It is possible, although largely speculative, that the glia-neuron trafficking of clusterin could be involved in a phenomenon like this whose mechanisms are unknown.

The present work adds a novel link between neurodegeneration and protein aggregation (Kakizuka, 1998) because clusterin, which is regularly found accumulated in neurodegenerative disorders, can also be strongly pro-aggregative depending on the clusterin fragments present. Contradictory activities of extracellular brain clusterin have been reported. Based on its solubilizing action observed in vitro (Matsubara et al., 1996; McHattie and Edington, 1999), clusterin is believed to inhibit neurotoxic agents but, conversely, the presence of clusterin in amyloid deposits suggests its inability to reverse protein precipitation. Moreover, studies of transgenic mice have suggested that clusterin expression promotes amyloid plaque formation in vivo (DeMattos et al., 2002). The biological action of intracellular clusterin, which is particularly abundant in certain neurons (Han et al., 2001; McGeer et al., 1992; Sasaki et al., 2002b; Senut et al., 1992) remains unknown, but our observations rather suggest a cell deleterious activity. One must notice that clusterin accumulation is often associated with intracellular protein aggregation. For example, cerebral ischemia, which leads to a strong intraneuronal accumulation of clusterin (Han et al., 2001), is associated with intense protein aggregation (Hu et al., 2000). Clusterin gene expression is also induced in retinitis pigmentosa (Jones and Jomary, 2002) and sporadic amyotrophic lateral sclerosis (Grewal et al., 1999), which are associated with the abnormal folding and aggregation of rhodopsin and oxidized superoxide dismutase, respectively (Rakshit et al., 2002; Saliba et al., 2002). The functional relationships between clusterin and intracellular protein aggregation will be of major importance to elucidate its precise participation to neurodegeneration.

This work was supported by grants from the Ligue Regionale Grand Ouest Contre le Cancer (LNCC), Association pour la Recherche Contre les Tumeurs de la Prostate (ARTP) and Association pour la Recherche Contre le Cancer (ARC). We are grateful to Catherine Martin for her outstanding technical assistance.

References

Bailey, R. W., Dunker, A. K., Brown, C. J., Garner, E. C. and Griswold, M. D. (2001). Clusterin, a binding protein with a molten globule-like region. Biochemistry 40, 11828-11840.

Buttyan, R., Olson, C. A., Pintar, J., Chang, C., Bandyk, M., Ng, P.-Y. and Sawczuk, I. S. (1989). Induction of the TRPM-2 gene in cells undergoing programmed death. Mol. Cell. Biol. 9, 3473-3481.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. (1989). A multiiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 243, 1576-1583.

Chung, K. K., Dawson, V. L. and Dawson, T. M. (2001). The role of the ubiquitin-proteasomal pathway in Parkinson’s disease and other neurodegenerative disorders. Trends Neurosci. 24, S7-S14.

Cyr, D. M., Hohfeld, J. and Patterson, C. (2002). Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. Trends Biochem. Sci. 27, 368-375.

Danik, M., Chabot, J.-G., Hassan-Gonzalez, D., Suh, M. and Quirion, R. (1993). Localization of sulfated glycoprotein-2/clusterin mRNA in the rat brain by in situ hybridization. J. Comp. Neurol. 334, 209-227.

DeMattos, R. B., O’dell, M. A., Parsadanian, M., Taylor, J. W., Harmony, J. A., Bales, K. R., Paul, S. M., Aronow, B. J. and Holtzman, D. M. (2002). Clusterin promotes amyloid plaque formation and is critical for neurotic toxicity in a mouse model of Alzheimer’s disease. Proc. Natl. Acad. Sci. USA 99, 10843-10848.

Desagher, S. and Martinou, J. C. (2000). Mitochondria as the central control point of apoptosis. Trends Cell. Biol. 10, 369-377.

Dragunow, M., Preston, K., Dodd, J., Young, D., Lawlor, P. and Christie, D. (1995). Clusterin accumulates in dying neurons following status epilepticus. Mol. Brain Res. 32, 279-290.

Duguid, J. R., Boehmert, C. W., Liu, C. W. and Tourtellote, W. W. (1989). Changes in brain gene expression shared by scrapie and Alzheimer disease. Proc. Natl. Acad. Sci. USA 86, 7260-7264.

Dul, J. L., Davis, D. P., Williamson, E. K., Stevens, F. J. and Argon, Y. (2001). Hsp70 and antibifilobogenic peptides promote degradation and inhibit intracellular aggregation of amyloidogenic light chains. J. Cell Biol. 152, 705-716.

Fabunmi, R. P., Wigley, W. C., Thomas, P. J. and DeMartino, G. N. (2000). Activity and regulation of the centrosome-associated proteasome. J. Biol. Chem. 275, 409-413.

Falnes, P. O. and Sandvig, K. (2000). Penetration of protein toxins into cells. Curr. Opin. Cell. Biol. 12, 407-413.

Cytokine antagonists: preclinical data and potential clinical uses. J. Leukoc. Biol. 39, 127-133.
McCarthy, N. J., Whyte, M. K. B., Gilbert, C. S. and Evan, G. I. (1997). Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or Bcl-2 homologue Bak. J. Cell Biol. 136, 215-227.

McGeer, P. L., Kawamata, T. and Walker, D. G. (1992). Distribution of clusterin in Alzheimer brain tissue. Brain Res. 579, 337-341.

McHattie, S. and Edington, N. (1999). Clusterin prevents aggregation of neurotope 106-126 in vitro. Biochem. Biophys. Res. Commun. 259, 336-340.

Michel, D., Chabot, J.-G., Moyse, E., Danik, M. and Quirion, R. (1992). Possible functions of a new genetic marker in central nervous system: the sulfated glycoprotein-2 (SGP-2). Synapse 11, 105-111.

Michel, D., Moyse, E., Brun, G. and Jourdan, F. (1994). Induction of apoptosis in mouse olfactory neuroepithelium by synaptic target ablation. Neuron 5, 1329-1332.

Michel, D., Chatelin, G., North, S. and Brun, G. (1997a). Stress-induced transcription of the clusterin/apoJ gene. Biochem. J. 328, 45-50.

Michel, D., Moyse, E., Trembleau, A., Jourdan, F. and Brun, G. (1997b). Clusterin/ApoJ expression is associated with neuronal apoptosis in the olfactory mucosa of the adult mouse. J. Cell. Sci. 110, 1635-1645.

Multhoff, G. and Hightower, L. E. (1996). Cell surface expression of heat shock proteins and the immune response. Cell Stress Chaperones 1, 167-173.

Newman, G. P., Wegryn, R. D., Lindquist, S. L. and Chernoff, Y. O. (1999). Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. Mol. Cell. Biol. 19, 1325-1333.

Norman, D. J., Feng, L., Cheng, S. S., Gubbay, J., Chan, E. and Heintz, N. (1995). The lurcher gene induces apoptotic death in cerebellar Purkinje cells. Development 121, 1183-1193.

Pasinetti, G. M., Johnson, S. A., Ota, T., Rozovsky, I. and Finch, C. E. (1994). Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain. J. Comp. Neurol. 339, 387-400.

Poon, S., Easterbrook-Smith, S. B., Rybchyn, M. S., Carver, J. A. and Wilson, M. R. (2000). Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-resistant state. Biochemistry 39, 15953-15960.

Poon, S., Treweek, T. M., Wilson, M. R., Easterbrook-Smith, S. B. and Carver, J. A. (2002a). Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. FEBS Lett. 513, 259-266.

Poon, S., Rybchyn, M. S., Easterbrook-Smith, S. B., Carver, J. A., Pankhurst, G. J. and Wilson, M. R. (2002b). Mildly acidic pH activates the extracellular molecular chaperone clusterin. J. Biol. Chem. 277, 39532-39540.

Poot, M., Zhang, Y. Z., Kramer, J. A., Wells, K. S., Jones, L. J., Hanzel, D. K., Lugade, A. G., Singer, V. L. and Haugland, R. P. (1996). Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J. Histochem. Cytochem. 44, 1363-1373.

Rakhit, R., Cunningham, P., Furtos-Matei, A., Dahan, S., Qi, X. F., Crow, J., Cashman, N. R., Kondejewski, L. H. and Chakrabartty, A. (2002). Oxidation-induced misfolding and aggregation of superoxide dismutase and its implications for amyotrophic lateral sclerosis. J. Biol. Chem. 277, 47551-47556.

Reddy, K. B., Jin, G., Karode, M. C., Harmony, J. A. and Howe, P. H. (1996). Transforming growth factor β (TGFβ)-induced nuclear localization of Apolipoprotein J/Clusterin in epithelial cells. Biochemistry 35, 6157-6163.

Sakahira, H., Breuer, P., Hayer-Hartl, M. K. and Hartl, F. U. (2002). Molecular chaperones as modulators of polyglutamine protein aggregation and toxicity. Proc. Natl. Acad. Sci. USA 99 Suppl. 4, 16412-16418.

Saliba, R. S., Munro, P. M., Luthert, P. J. and Cheetham, M. E. (2002). The cellular fate of mutant rhodopsin: quality control, degradation and aggroseome formation. J. Cell Sci. 115, 2907-2918.

Sasaki, K., Doh-Ura, K., Ironside, J. W. and Iwaki, T. (2002a). Increased clusterin (apolipoprotein J) expression in human and mouse brains infected with transmissible spongiform encephalopathies. Acta Neuropathol. 103, 199-208.

Sasaki, K., Doh-Ura, K., Wakisaka, Y. and Iwaki, T. (2002b). Clusterin/apolipoprotein J is associated with cortical Lewy bodies; immunohistochemical study in cases with alpha-synucleinopathies. Acta Neuropathol. 104, 225-230.

Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M. and Lee, C. (1995). Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by...
overexpression of sulfated glycoprotein-2 (clusterin). Cancer Res. 55, 2431-2437.
Senut, M. C., Jazat, F., Choi, N. H. and Lamour, Y. (1992). Protein SP40,40-like immunoreactivity in the rat brain – progressive increase with age. Eur. J. Neurosci. 4, 917-928.
Simpson, J. C., Roberts, L. M., Romisch, K., Davey, J., Wolf, D. H. and Lord, J. M. (1999). Ricin A chain utilises the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. FEBS Lett. 459, 80-84.
Sourisseau, T., Desbois, C., Debure, L., Bowtell, D. D., Cato, A. C., Schneikert, J., Moyse, E. and Michel, D. (2001). Alteration of the stability of Bag-1 protein in the control of olfactory neuronal apoptosis. J. Cell Sci. 114, 1409-1416.
Stieber, A., Gonatas, J. O. and Gonatas, N. K. (2000). Aggregates of mutant protein appear progressively in dendrites, in periaxonal processes of oligodendrocytes, and in neuronal and astrocytic perikarya of mice expressing the SOD1(G93A) mutation of familial amyotrophic lateral sclerosis. J. Neurol. Sci. 177, 114-123.
Tamaoka, A., Mizusawa, H., Mori, H. and Shoji, S. (1995). Ubiquitinated αB-crystallin in glial cytoplasmic inclusions from the brain of a patient with multiple system atrophy. J. Neurol. Sci. 129, 192-198.
Tytell, M., Greenberg, S. G. and Lasek, R. J. (1986). Heat shock-like protein is transferred from glia to axon. Brain Res. 363, 161-164.
Vayssière, J. L., Petit, P. X., Riser, Y. and Mignotte, B. (1994). Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with Simian Virus 40. Proc. Natl. Acad. Sci. USA 91, 11752-11756.
Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H. and Waneker, E. E. (2001). Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol. Biol. Cell 12, 1393-1407.
Walton, M., Young, D., Sirimanne, E., Dodd, J., Christie, D., Williams, C., Gluckman, P. and Dragunow, M. (1996). Induction of clusterin in the immature brain following a hypoxic-ischemic injury. Mol. Brain Res. 39, 137-152.
Wehrli, P., Charnay, Y., Vallet, P., Zhu, G., Harmony, J., Aronow, B., Tschopp, J., Bouras, C., Viard-Leveugle, I., French, L. E. et al. (2001). Inhibition of post-ischemic brain injury by clusterin overexpression. Nat. Med. 7, 977-978.
Wilson, M. R. and Easterbrook-Smith, S. B. (2000). Clusterin is a secreted mammalian chaperone. Trends Biochem. Sci. 25, 95-98.
Wyttenbach, A., Carmichael, J., Swartz, J., Furlong, R. A., Narain, Y., Rankin, J. and Rubinsztain, D. C. (2000). Effects of heat shock, heat shock protein 40 (HDI-2), and proteasome inhibition on protein aggregation in cellular models of Huntington`s disease. Proc. Natl. Acad. Sci. USA 97, 2898-2903.
Yang, C. R., Leskov, K., Hosley-Eberlein, K., Criswell, T., Pink, J. J., Kinsella, T. J. and Boothman, D. A. (2000). Nuclear clusterin/XIP8, an X-ray-induced Ku70-binding protein that signals cell death. Proc. Natl. Acad. Sci. USA 97, 5907-5912.