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Alterations in the post-translational modification and intracellular trafficking of clusterin in MCF-7 cells during apoptosis

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Abstract

Clusterin is a heterodimeric, disulfide-linked 70–80 kDa glycoprotein that is induced during regression of most, if not all, hormone-dependent epithelial tissues. These studies describe the biogenesis and intracellular trafficking of clusterin in MCF-7 cells before and after the initiation of apoptosis with antiestrogens and TNFα. Under physiological conditions, clusterin is modified in the endoplasmic reticulum (ER), and proteolytically cleaved in the Golgi to generate discrete α and β chains prior to secretion. Treatment with TNFα or the antiestrogen, ICI 182,780, induces apoptosis in MCF-7 cells and leads to substantial changes in the activity of Golgi-resident enzymes, significantly altering the biogenesis of clusterin. This leads to the appearance of a 50–53 kDa uncleaved, nonglycosylated, disulfide-linked isoform of clusterin that accumulates in the nucleus. While clusterin contains a cryptic SV-40-like nuclear localization signal, mutation of this sequence does not affect the nuclear accumulation of the disulfide-linked nuclear isoform. Confocal microscopy demonstrates that the nuclear accumulation of clusterin is coincident with DNA fragmentation. These data suggest that, at least in secretory epithelial cells, retrograde transport from the Golgi to the ER of a nonglycosylated, uncleaved isoform and the subsequent translocation of clusterin to the nucleus occur in dying cells.

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Abbreviations: ER, endoplasmic reticulum; BFA, Brefeldin A; FFE, free flow electrophoresis; Gal T, galactosyl transferase; Mann II, mannosidase II; NLS, nuclear localization signal; OST, oligosaccharide transferase.

Introduction

Epithelial cell death has been characterized in the secretory epithelium of the regressing breast after the ablation of the appropriate trophic hormones or administration of antiestrogens,[1] and in the prostate after castration or administration of antiandrogens.[2,3] One of the most prominent proteins induced during the regression of the rat ventral prostate or mammary gland is clusterin.[1,4–8] Clusterin is encoded by a single copy gene located on chromosome 8, just distal to the transition between 8p12 and 8p21,[9,10] and chromosome 14 in the mouse.[11,12] The gene has been isolated and sequenced from a number of species including human,[10] rat[6] and mouse,[12] and has a similar structure of nine exons and eight introns, spanning approximately 14 kb in the rat and 17 kb in the human. Primer extension analysis has shown that there is a single functional promoter in the rat and human genes.[6,10] Transcription of the clusterin gene gives rise to a single mRNA, 1.6 kb in length, which is translated on membrane-bound ribosomes[13] and, like other proteins containing a hydrophobic leader sequence, is cotranslationally translocated to the lumen of the endoplasmic reticulum (ER) coincident with the cleavage of the signal peptide. Subsequent glycosylation in the (ER) and Golgi, and proteolytic cleavage of the protein between R205 and S206 produce a mature protein of 427 amino acids that is a secreted, disulfide-bonded, heterodimeric glycoprotein containing different but similarly sized α and β chains.[14] (The numbering of the amino acids, and the position of the cleavages sites are based on the mature protein.) In the human, there are six N-linked glycosylation sites, three on the α chain (α64N, α81N and α123N) and three on the β chain (β64N, β127N and β147N), all of which appear to be utilized.[14,15] Clusterin also contains a cryptic SV-40-like nuclear localization signal (NLS) (SNLEEAKKKK), located between amino acids 50 and 60 of the mature protein. However, under normal homeostatic conditions, this localization sequence is not utilized since clusterin is translated on bound ribosomes and the protein is secreted.

Clusterin was first associated with cell death in the regressing rat ventral prostate, but has since been identified as an induced gene in numerous *in vivo* and *in vitro* models of epithelial cell death including the endometrium,[16] kidney,[17,18] pancreas,[19] brain[20–22] and retinal tissue.[23] While clusterin expression is clearly induced very significantly during cell death in these systems, it is also clear that clusterin is

expressed constitutively in many adult organs in physiological circumstances that are not associated with apoptosis, most prominently in the testes,[24,25] and complement system.[26] In general, expression is spatially restricted to the epithelial compartment,[3,27,28] and is found at significant levels in many extracellular fluids including blood,[29] cerebral spinal fluid,[30] and seminal fluid.[31] However, in some cell types with a regulated exocytic pathway, including platelets,[22,32] and in endocrine and neuroendocrine secretory tissues[33,34] clusterin is stored in secretory granules and released upon stimulated exocytosis. In these contexts, it has been suggested that clusterin may play a role in cell survival.

Few studies have directly examined the effects of experimentally manipulating levels of clusterin expression on cell death and survival. In human prostatic LNCaP cells, overexpression of clusterin provides protection against TNFα-induced cell death and oligonucleotide directed antisense inhibition enhances spontaneous cell death in untreated cultures.[35] In L929 cells, overexpression of clusterin protects the cells against TNFα-induced cell death.[36] These data suggest that rather than being part of the cell death mechanism, as most of the *in vivo* data suggest, clusterin may have a cytoprotective role in epithelial cell death. Thus, it is unclear whether clusterin is directly involved in the apoptotic process, whether its induction is secondary to apoptosis, or whether the protein is induced as a protective or survival mechanism for the cell.

To address the issues relating to the functional role of clusterin in apoptosis we have used a monoclonal antibody raised against rat recombinant clusterin that recognizes both the wild-type clusterin and a unique ''apoptosis-related'' isoform of the protein.[37] We have characterized the biogenesis of clusterin in MCF-7 cells by free flow electrophoresis (FFE), Western analysis and immunohistochemistry, both before and after treatment with TNFα or the pure antiestrogen ICI 182,780.[38] Both of these compounds induce apoptotic cell death in MCF-7 cells in a dose- and time-dependent manner. TNFα utilizes the extrinsic cell death pathway, while ICI 182,780 disrupts estrogen receptor-mediated signaling required for cell survival. The data demonstrate that there are significant alterations in the biogenesis of clusterin during apoptosis, which lead to the appearance of a nonglycosylated, disulfide-linked isoform in the nucleus of those cells which initiate DNA fragmentation.

Results

Inhibition of cell proliferation and induction of apoptosis in MCF-7 cells

The effects of TNFα, ICI 182,780 and Brefeldin A (BFA) on the growth kinetics of MCF-7 cells *in vitro* are shown in Figure 1. All the three drugs induce growth inhibition and apoptosis in a time dependent manner. The induction of apoptosis was monitored by changes in cellular morphology, TUNEL analysis and DNA fragmentation (data not shown). The induction of apoptosis by ICI 182,780 is time- and dose-dependent. Doses of 10 μM ICI182,780 induce growth arrest and apoptosis that is first visible at 24 h, but is much more prominent at 96 h (Figure 1, panels a and b). MCF-7 cells are also sensitive to TNFα doses as low as 1 ng/ml. When treated with this concentration of TNFα, MCF-7 cells display 40–50% reduction in cell number as early as 24 h, and this percentage increases to 85–90% by 72 h of incubation (Figure 1, panels c and d). There is clear evidence of DNA fragmentation, as measured by TUNEL staining as early as 24 h after TNFα treatment (results not shown). In marked contrast, to both TNFα and ICI 182,780, the fungal antibiotic, BFA, which induces rapid regression of the Golgi stack into rudimentary Golgi clusters in parallel with a redistribution of the Golgi-resident proteins into the rough ER, at doses of 5 μg/ml induce 70–80% of the cells to undergo cell death by 6 h (Figure 1, Panel e), although there is very little evidence of DNA fragmentation at this time in cells treated with BFA (results not shown).

FFE analysis of clusterin biogenesis

For FFE experiments, MCF-7 cells were treated with 1 ng/ml TNFα, or 10 μM ICI 182,780, and harvested 24 h later, at which time the majority of the cells are in the early stages of apoptosis, prior to the formation of apoptotic bodies. The cells were harvested 6 h after BFA treatment. In untreated MCF-7 cells, marker enzyme analysis of purified subfractions from FFE indicates that organelles emerge mostly as single asymmetrical peaks, separated into different subfractions based on membrane surface charge and size (Figure 2, panel a). Based on marker enzyme profiles, the plasma membrane fraction is restricted to fractions 40–43, lysosomal membranes are found in fractions 30–33, the Golgi apparatus is concentrated in fractions 35–39 and the ER is restricted to fractions 40–43. To determine the subcellular location associated with the post-translational modification of clusterin, the FFE fractions were concentrated, immunoblotted and probed with 6E9, the anticlusterin monoclonal antibody that recognizes both the wild-type clusterin and the putative apoptotic glyco/isoform of clusterin.[37] In untreated MCF-7 cells, a 50–53 kDa isoform of clusterin is present in the ER/plasma membrane fraction (Figure 2, panel b). Since clusterin is known to be a secreted protein and is not localized on the membrane by immunohistochemistry, this isoform of clusterin most likely represents an isoform localized to the ER. In the Golgi fraction, the β-chain-specific monoclonal, 6E9, detects a 29 kDa clusterin polypeptide, the product of the endopeptidase cleavage of clusterin into α and β chains. There is no evidence of clusterin in the lysosomal fraction, and clusterin is secreted into the medium where it is detectable by Western analysis (data not shown). After treatment with 1 ng/ml TNFα, there are significant, reproducible changes in the marker enzyme profiles and in the biogenesis of clusterin (Figure 3, panel a). While marker enzyme profiles for the ER, lysosomes and plasma membrane remain essentially unaltered, mannosidase II (mann II) activity is not detectable in the FFE fractions. However, after concentrating fractions 35–39 from FFE runs, and probing with the 6E9 monoclonal anticlusterin antibody, a 50–53 kDa isoform clusterin is clearly detected in these fractions and in the fractions containing the Golgi apparatus (Figure 3, panel b). These data suggest that the internal proteolytic cleavage of clusterin in the Golgi apparatus is blocked after treatment with TNFα. Similar changes in the

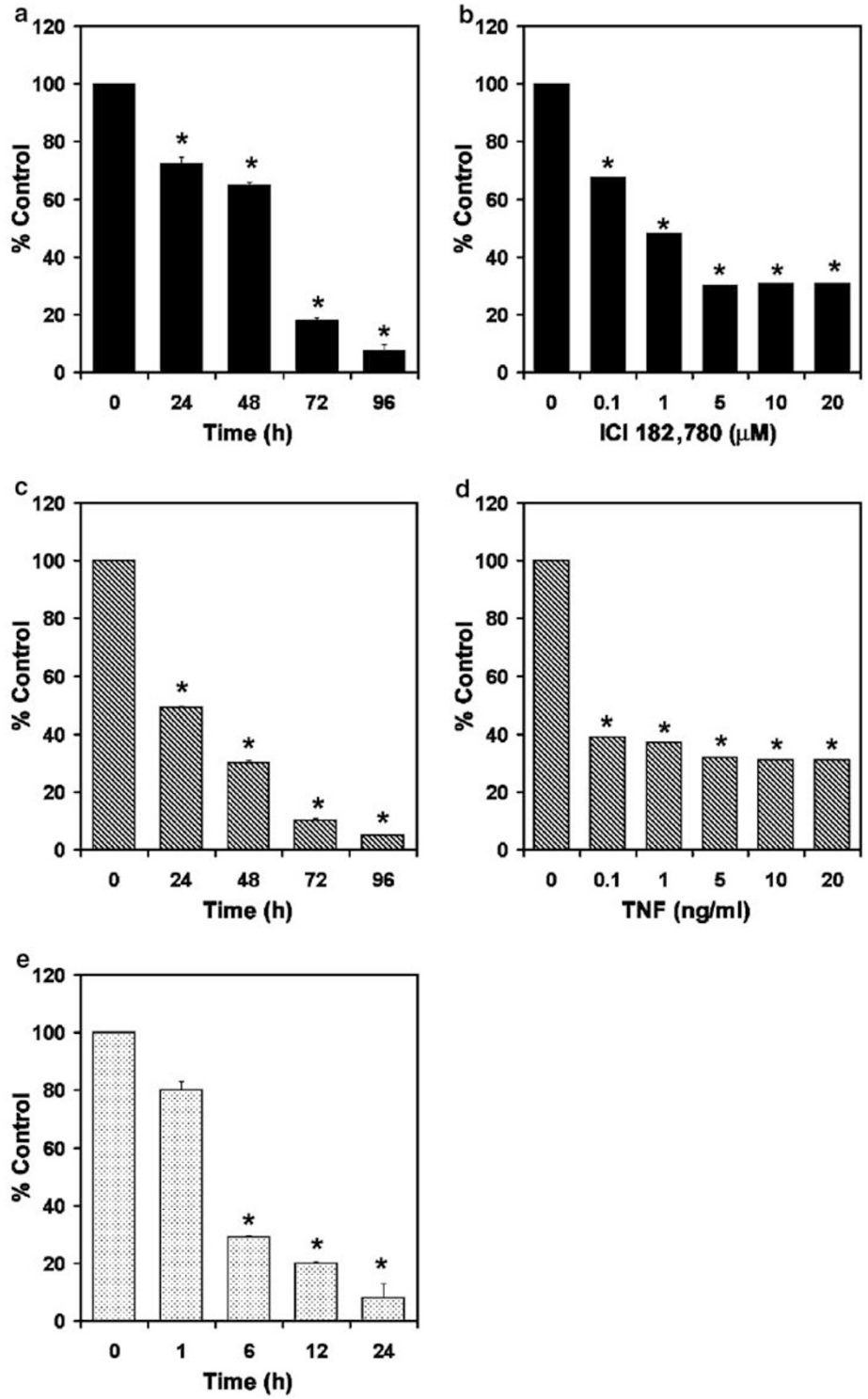


Figure 1 Growth kinetics of MCF-7 cells after treatment with TNFα, ICI 182,780 or BFA. Time course (**a**, **c**, **e**) and dose response (**b**, **d**) of MCF-7 cells to ICI 182,780 (**a**, **b**), TNFα (**c**, **d**) or BFA (**e**). (**a**, **c**, **e**) 2×10^4 MCF-7 cells, plated in 24-well plates were treated in phenol red-free α-MEM supplemented with 5% CSS and 10 nM 17β-estradiol with 10 μM ICI 182,780, 1 ng/ml TNFα or 5 μg/ml BFA, respectively, for the indicated times. Cell numbers were assayed using crystal violet as described in Materials and Methods. (**b**, **d**) 2×10^4 MCF-7 cells, plated in 24-well plates were treated in phenol red-free α-MEM supplemented with 5% CSS with increasing doses of ICI 182,780 or TNFα for 72 h. Untreated MCF-7 cells served as the control at each time point. Results are expressed as mean $\pm$ S.D. of three independent experiments. $^*P < 0.05$.

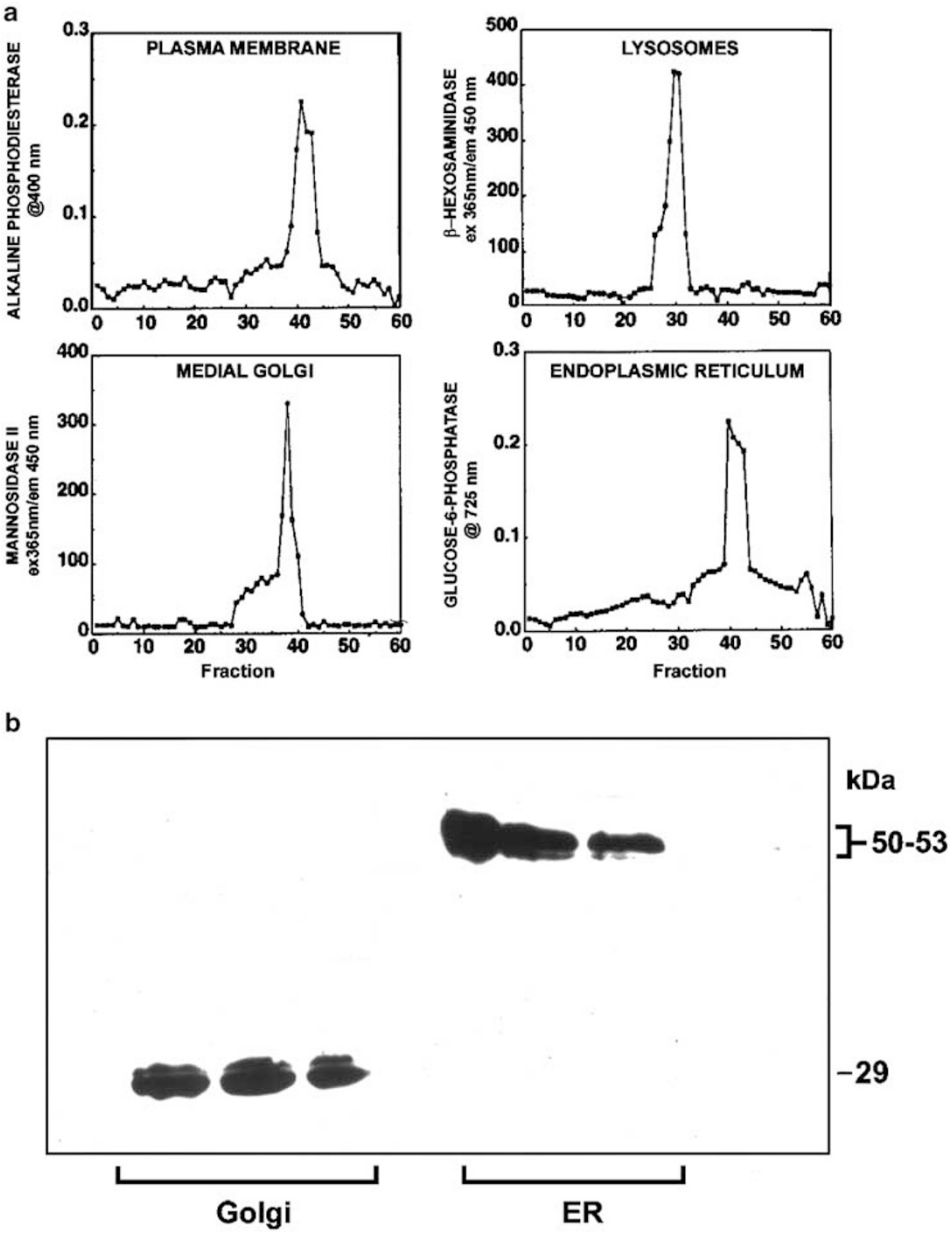
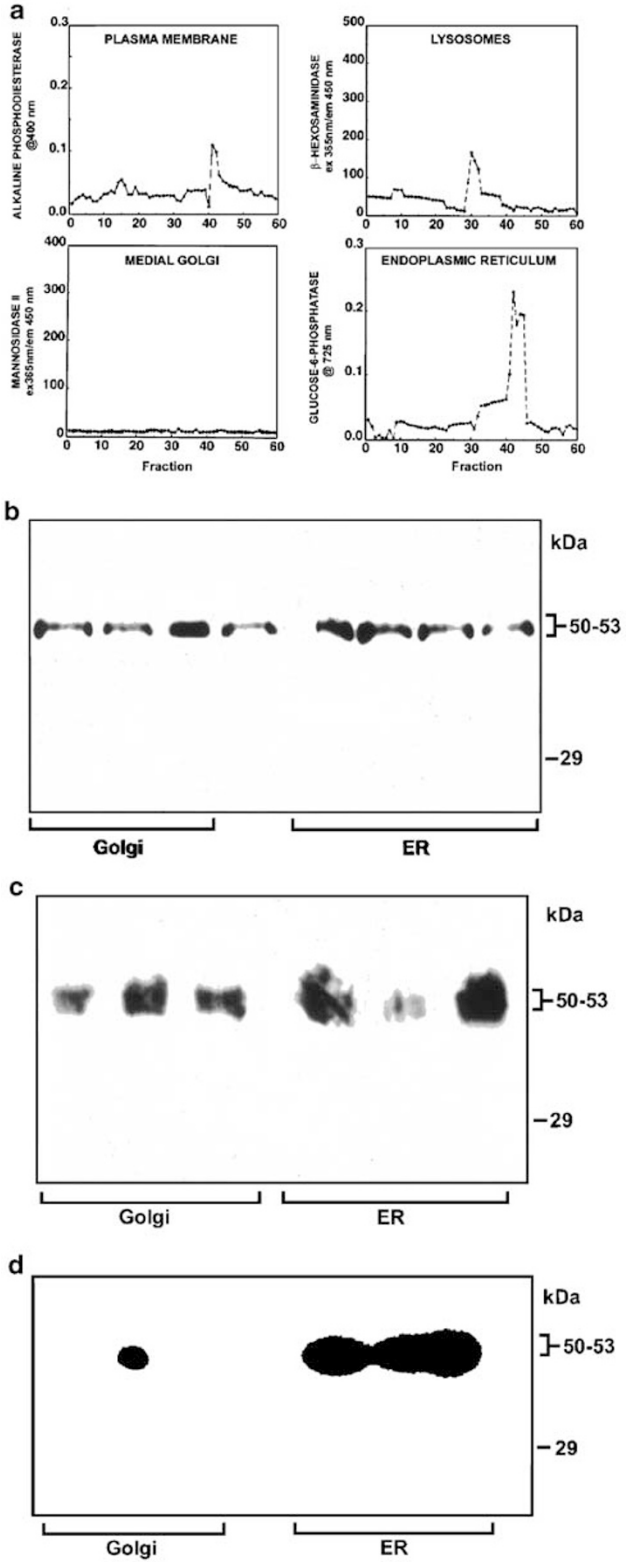


Figure 2 Analysis of intracellular processing of clusterin in untreated MCF-7 cells using FFE. (**a**) Marker enzyme profiles for untreated MCF-7 cells. FFE fractions were collected and assayed for marker enzyme activities as described in Materials and Methods. (**b**) Intracellular processing of clusterin. FFE fractions were collected, concentrated and electrophoresed on reducing 12.5% SDS-PAGE. The proteins were transferred to nitrocellulose membranes, probed with 6E9, the anticlusterin antibody as described in Materials and Methods. The ER (fractions 41, 42 and 43) contains a 50–53 kDa intermediate precursor, which is proteolytically cleaved in the Golgi apparatus (fractions 36, 37 and 38) to produce the 29 kDa β chain that is detected by 6E9.

biogenesis of clusterin are seen when the cells are treated with 10 μM ICI 182,780 (Figure 3, panel c), although the loss of mann II enzymatic activity is not as pronounced after treatment with the pure antiestrogen (data not shown). The secretion of clusterin is also reduced, but not eliminated, after treatment with both these apoptotic inducers (data not shown). These changes in clusterin biogenesis demonstrate that the proteolytic cleavage required to produce the mature secretory form of the protein does not occur in the Golgi after treatment with either TNFα or ICI 182,780.

When treated with 5 μg/ml BFA, clusterin secretion from MCF-7 cells is inhibited in a time-dependent manner (data not shown). Furthermore, FFE and Western analysis demonstrate that clusterin is inefficiently transported to the Golgi, and is not processed by proteolysis (Figure 3, Panel d). Thus, while BFA clearly exerts a very significant effect on the proteolytic conversion of clusterin to the mature α and β chains, it does so primarily by blocking the anterograde transport of clusterin from the endoplasmic reticulum (ER) to the Golgi, where terminal glycosylation of oligosaccharide

chains in glycoproteins and proteolytic processing of clusterin takes place. Thus, although treatment with TNFα and ICI 182,780 also results in the failure to cleave clusterin proteolytically, the effect appears to be directly related to the proteolytic processing itself, since clusterin is translocated to the Golgi.



Expression of mann II and galactosyl transferase (Gal T) in MCF-7 cells after treatment with TNFα or ICI 182,780

MannII is a 124–130 kDa glycoprotein that is normally resident in the cis/medial Golgi. It is synthesized as a glycoprotein and is modified during transit through the ER to the Golgi. This modification varies slightly from cell type to cell type resulting in variation in the size of glycoprotein.[39] To determine whether the loss of mann II enzymatic activity seen in the purified FFE fractions is reflective of generalized changes in the expression of Golgi proteins or because of the fractionation procedure, we examined the expression of this enzyme in cytosolic extracts of MCF-7 cells after treatment with TNFα or ICI 182,780. The level of mann II cytosolic protein decreases rapidly after treatment with 1 ng/ml TNFα (Figure 4, panel a), and mann II is barely detectable in MCF-7 cells 48 h after treatment with TNFα. Immunofluorescent studies using the same antibody, confirm the dramatic decrease in the expresssion of mann II 48 h after treatment (Figure 4, panel a). The expression of mann II in untreated MCF-7 cells is diffuse, while after treatment with TNFα, the structure of the Golgi apparatus appears to alter, as indicated by the different staining pattern of mann II surrounding the nucleus (Figure 4, panel b). In particular, the level of mann II staining in the Golgi varies noticeably among cells observed at the same time after treatment, suggesting that the Golgi apparatus undergoes a progressive inactivation and/or disruption after TNFα treatment. A similar reduction in the level of mann II expression is detected after treatment with 10 μM ICI 182,780, both by Western analysis and by immunofluorescence (Figure 4, panel b). However, the time frame of the changes in mann II expression is considerably more extended than seen with TNFα and the morphological disruption is not as dramatic. No reduction in the expression levels of mann II is detected after BFA treatment (data not shown).

The changes in mann II activity after treatment with TNFα and ICI 182,780 are reflected in the processing of galactosyl transferase (Gal T). Prior to treatment, MCF-7 cells express the mature 45 kDa form of Gal T (Figure 4, panel c). At 48 h after TNFα treatment, there is a significant decrease in the

Figure 3 Intracellular processing of clusterin after TNFα or ICI182,780 or BFA treatments. (**a**) Marker enzyme profiles for MCF-7 cells treated with TNFα (1 ng/ml) for 24 h using FFE. FFE fractions were collected and assayed for marker enzyme activities as described in Materials and Methods. (**b**) Intracellular processing of clusterin in the ER and the Golgi apparatus after treatment with 1 ng/ml TNFα for 24 h. FFE fractions were collected, concentrated and electrophoresed on 12.5% SDS PAGE, samples containing 2.5% β-mercaptoethanol. The proteins were transferred to nitrocellulose membranes, probed with 6E9 as described in Materials and Methods. Clusterin is present in the ER and Golgi as a 50–53 kDa intermediate precursor, indicating that internal proteolytic cleavage, to produce the 29 kDa β chain, does not occur. (**c**) Intracellular processing of clusterin in the ER and the Golgi apparatus after treatment with 10 μM ICI 182,780 for 24 h. There is no evidence of proteolytic cleavage. (**d**) Intracellular processing of clusterin in the ER and the Golgi apparatus after treatment with 5 μg/ml BFA for 6 h. Clusterin is present in the ER as a 50–53 kDa intermediate precursor; however little, if any, clusterin is found in the Golgi apparatus.

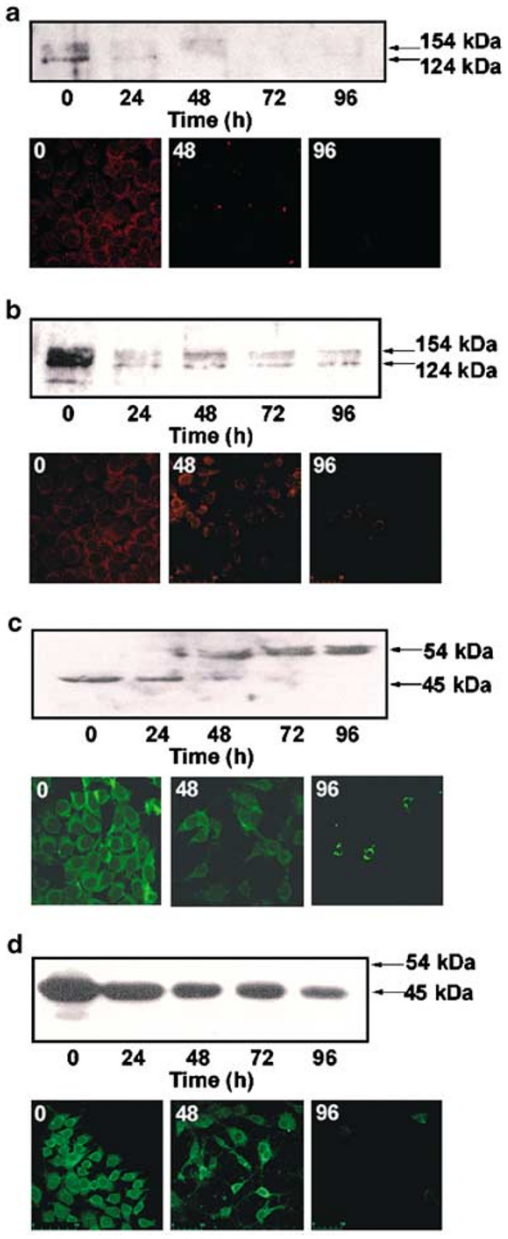


Figure 4 Expression of mann II and Gal T after treatment with TNFα or ICI 182,780 treatments. (**a**) MCF-7 cells were treated for the indicated times with 1 ng/ml TNFα and processed for Western analysis (upper panel) or immunofluorescence (lower panel) using polyclonal antibody specific for mann II, as described in Materials and Methods. (**b**) MCF-7 cells were treated for the indicated times with 10 μM ICI 182,780 and processed for Western analysis (upper panel) or immunofluorescence (lower panel) using a polyclonal antibody specific for mann II, as described in Materials and Methods. (**c**) MCF-7 cells were treated for the indicated times with 1 ng/ml TNFα and processed for Western analysis or immunofluorescence using a polyclonal antibody specific for Gal T, as described in Materials and Methods. (**d**) MCF-7 cells were treated for the indicated times with 10 μM ICI 182,780 and processed for Western analysis or immunofluorescence using a polyclonal antibody specific for Gal T as described in Materials and Methods. This blot was exposed for longer times to confirm that the 54 kDa band was not expressed.

level of the 45 kDa form, and the appearance of a higher molecular weight 54 kDa isoform. This isoform appears to accumulate as a result of the loss of mann II activity, and the concomitant failure to remove the terminal mannose residues from Gal T. After treatment of MCF-7 cells with ICI182,780, there is a steady decrease in the level of the mature isoform of Gal T; however, the higher molecular weight isoform is not detected even after extended exposure times (Figure 4, panel d), probably because of the continued presence of sufficient mann II in the medial Golgi (see Figure 4, panel b) to ensure appropriate post-translational cleavage of the terminal mannose residues in Gal T and other glycoproteins transiting the medial Golgi. This relation between the presence of mann II and the mature 45 kDa isoform of Gal T is further illustrated after treatment with BFA. At 6 h after treatment of MCF-7 cells with BFA, both the 54 and 45 kDa isoforms are present in the cell, while at 12 h after treatment, only the mature 45 kDa isoform is evident, presumably because of the continuing activity of mann II (results not shown).

Translocation of clusterin to the nucleus in MCF-7 cells after treatment with TNFα and ICI 182,780.

The changes in the processing of clusterin in the Golgi lead to a decrease in the secretion of the protein (data not shown); however, there is a concomitant accumulation of a 50–53 kDa isoform in the nucleus 72–96 h after treatment of MCF-7 cells with either TNFα or ICI 182,780 (Figure 5, panels a and b). This isoform accumulates in the nucleus between 72 and 96 h after treatment with either TNFα or the antiestrogen. This is not because of ER contamination of the nuclear fractions, since there is no appreciable contamination of glucose-6-phosphatase (the marker for the ER) in the nuclear preparations (Figure 5, panel c and d). In contrast, there is no nuclear accumulation of clusterin after BFA treatment (data not shown). Since clusterin is secreted by both normal and dying cells, it is difficult to quantitate accurately the proportion of the protein that is redirected to the nucleus in dying cells. However, based on Western analysis of concentrated medium and nuclear extracts, it appears that between 5 and 15% of the total clusterin synthesized is localized to the nucleus of adherent cells.

The nuclear isoform of clusterin isolated from MCF-7 cells treated with either 1 ng/ml TNFα or 10 μM ICI 182,780 does not appear to contain any N-linked oligosaccharides since treatment with PNGase F does not alter the electrophoretic mobility of the protein (Figure 6, panel a). However, clusterin isolated from the nucleus after treatment with either TNFα or ICI 182,780 does appear to be disulfide-linked since the electrophoretic mobility is significantly altered by the addition of β-mercaptoethanol (Figure 6, panels b and c). The additional bands in the nonreduced lanes are most likely because of the presence of small amounts of reducing reagents in the initial homogenization buffer.

To determine whether the nuclear isoform of clusterin is expressed predominantly in surviving or dying cells, clusterin and TUNEL staining were performed on the same cells. Colocalization studies in MCF-7 cells using TUNEL staining and the 6E9 anticlusterin monoclonal antibody demonstrate that after treatment with TNFα or ICI 182,780 for 72 h, nuclear clusterin is exclusively localized to cells that have initiated DNA fragmentation (Figure 7, Panel a). In cells treated with either TNFα or ICI 182,780, clusterin levels are upregulated

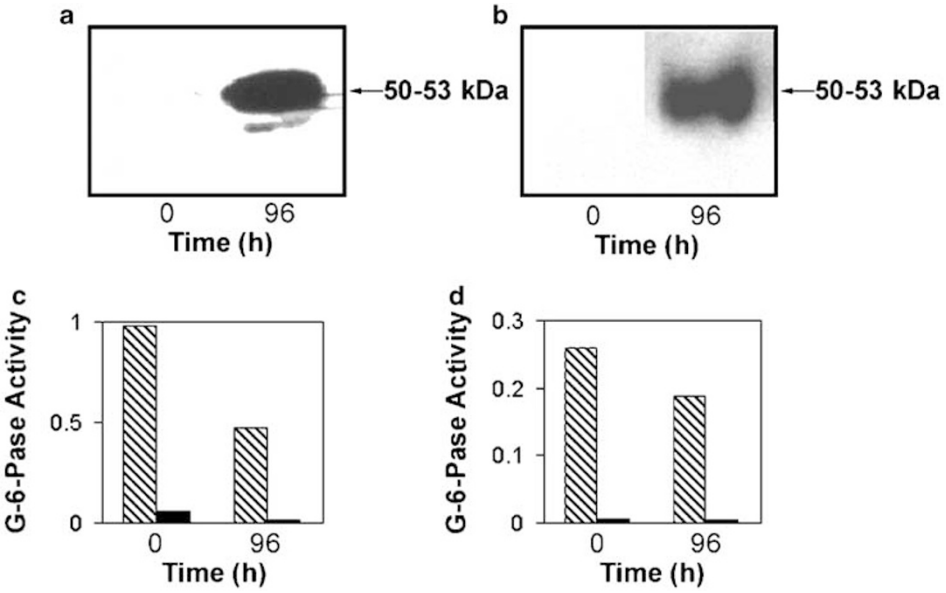


Figure 5 Characterization of the nuclear isoform of clusterin. MCF-7 cells were treated for 96 h with 1 ng/ml TNFα (panels **a**, **c**) or 10 μM ICI 182,780 (**b**, **d**). (**a** and **b**) Aliquots of the nuclear fractions were electrophoresed on 12.5% SDS-PAGE gels, transferred to nitrocellulose membrane and probed with the anticlusterin monoclonal antibody 6E9. (**c**, **d**) Protein extracts from postnuclear supernatants (hatched bars) and nuclear pellets (solid bars) were prepared and assayed for glucose-6-phosphatase (the ER marker enzyme) to monitor contamination of the nuclear fraction by the ER as described in Materials and Methods.

and the localization of the protein in the cytoplasm becomes punctate, suggesting that the protein is accumulating within the endomembrane system. Furthermore, a significant proportion of the protein is localized in the nuclei, which also demonstrates extensive DNA cleavage. While all cells expressing nuclear clusterin also show evidence of DNA fragmentation, between 20 and 30% of the TUNEL-positive nuclei cells do not contain clusterin, regardless of the drug or dosage used (Figure 7, Panels b and c). This suggests that DNA fragmentation precedes the translocation of clusterin to the nucleus, and further that this translocation is not the trigger for DNA fragmentation.

Role of cryptic NLS in nuclear accumulation of clusterin during apoptosis

While these data provide strong evidence that a nonglycosylated isoform of clusterin accumulates in the nucleus of MCF-7 cells undergoing apoptosis after treatment with TNFα or ICI 182,780, they do not provide any indication of the mechanism leading to this accumulation. As described earlier, clusterin contains a perfect SV40-type NLS between amino acids 50

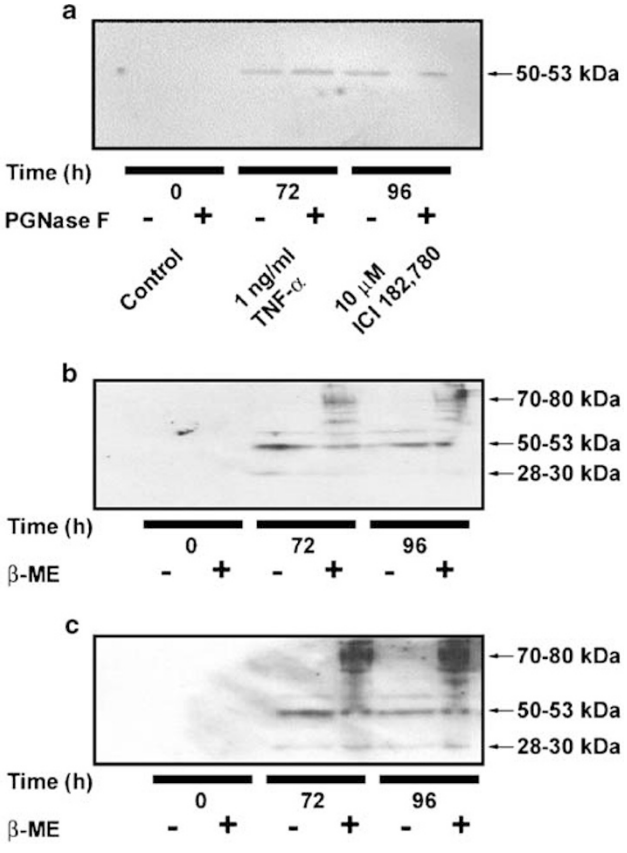


Figure 6 Effect of TNFα or ICI 182,780 on glycosylation and disulfide bond formation of the nuclear isoform of clusterin. (**a**) Glycosylation state of the nuclear isoform of clusterin. MCF-7 cells were treated with 1 ng/ml TNFα or 10 μM ICI 182,780 for 96 h and processed for Western analysis. Purified nuclear samples were treated with or without 1000 U PNGase F as described in Materials and Methods. No decrease in molecular weight was detected, indicating that the nuclear glyco/isoform of clusterin expressed during apoptosis is not glycosylated. (**b**, **c**) Disulfide bond formation in the nuclear isoform of clusterin. MCF-7 cells were treated for the indicated times with 1 ng/ml TNFα (**b**) or 10 μM ICI 182,780 (**c**). Purified nuclear fractions were prepared as outlined in Materials and Methods. Aliquots of the nuclear fractions were electrophoresed on 12.5% nondenaturing PAGE gels, transferred to nitrocellulose membrane and probed with the anticlusterin monoclonal antibody 6E9. Each time point sample was run in the absence or presence of 2.5% β-mercaptoethanol in the sample loading buffer.

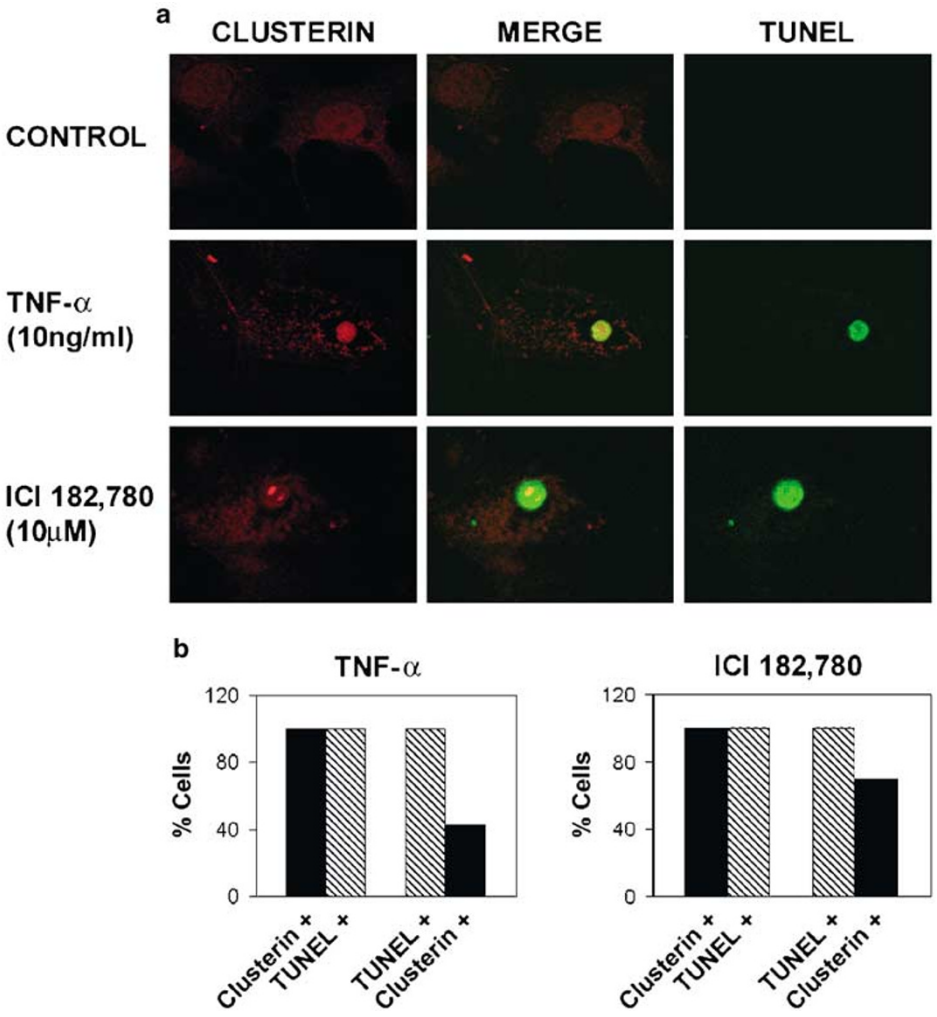


Figure 7 Localization of clusterin in the nuclei of apoptotic cells. (**a**) MCF-7 cells were treated with 1 ng/ml TNFα or 10 μM ICI 182,780 for 96 h, and fixed for TUNEL staining and immunofluorescence using the 6E9 anticlusterin monoclonal antibody and analyzed by confocal microscopy as described in Materials and Methods. Clusterin expression is visualized with 6E9 and Cy3-labeled secondary antibody. Fragmented DNA is visualized using TUNEL labeling with fluorescein-labeled nucleotides. Merged images were created using Confocal Assistant and Abode Photoshop. (**b**, **c**): Relation between clusterin-positive and TUNEL-positive cells in cells treated with 10 ng/ml TNFα (**b**) or 10 μM ICI 182,780 (**c**). Nuclear clusterin-positive cells (solid bars) were assessed for DNA fragmentation by TUNEL staining (hatched bars). TUNEL-positive cells were also assessed for nuclear clusterin expression.

and 60 on the mature α chain. During normal synthesis and secretion, this NLS is not normally utilized since the protein is translated on membrane-bound ribosomes, and is translocated to the lumen of the ER, where it is folded and glycosylated, effectively sequestering it away from the nuclear transport machinery. To determine whether the NLS is required for the translocation of clusterin to the nucleus after treatment with TNFα or antiestrogens, the NLS was mutated and the effects of this mutation on the nuclear localization after induction of apoptosis were examined. To distinguish the mutated proteins from the endogenous clusterin, the mutant construct was cloned into the pcDNA6 vector in frame with the myc epitope (Figure 8, panel a). Using a mouse monoclonal antibody specific for the myc epitope to detect the mutated clusterin, it is clear that clusterin containing the mutated NLS is still efficiently translocated to the nucleus after treatment with TNFα or ICI 182,780 treatment, suggesting that the NLS is not utilized for the relocalization of the protein to the nucleus after treatment with TNFα or ICI 182,780 (Figure 8, panel b).

Discussion

Clusterin was originally identified as a sulfated glycoprotein synthesized by the Sertoli cells of the testes, and was proposed to play a significant role in sperm maturation.[40,41] The same gene product was identified and cloned from the regressing rat ventral prostate and shown to be substantially induced in both the regressing prostate and mammary gland after hormone ablation or antihormone therapy.[3,4,8,42] The expression of clusterin in these physiological contexts of tissue regression suggests that the protein may play a role in apoptotic cell death. However, the expression of the protein in numerous pathophysiological systems that do not involve apoptotic cell death has raised doubts as to whether the protein is involved in apoptosis and it has been suggested that

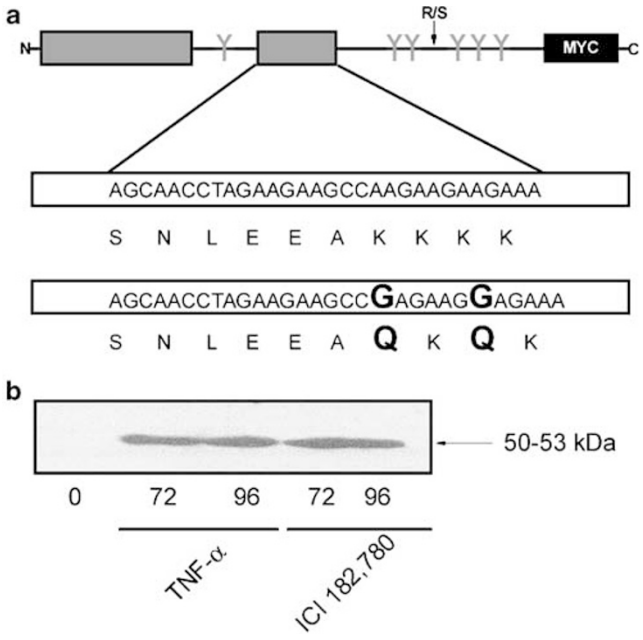


Figure 8 Nuclear localization of clusterin does not require the NLS. (**a**) Details of the point mutations in the NLS of clusterin. (**b**) MCF-7 cells, stably transfected with a clusterin construct containing the mutated NLS and containing a C-terminal myc epitope tag were treated with 10 ng/ml TNFα or 10 μM ICI 182,780 for the indicated times. Purified nuclear samples were prepared as described in Materials and Methods and Western blotted using an antibody specific for the myc epitope.

the protein may even confer a survival advantage.[28,43] This latter suggestion is further supported by the characterization of the secreted protein as an effective extracellular Type II chaperone that solubilizes partially denatured proteins.[44,45] There is considerable heterogeneity in the post-translational modification of clusterin, perhaps most dramatically illustrated by the testes-specific sulfation of the glycoprotein,[42,46] suggesting that the function of the glycoprotein may be altered by its post-translational modification.

The data presented in this manuscript demonstrate that the biosynthesis and post-translational modification of clusterin are dramatically altered in MCF-7 cells after the induction of cell death by either TNFα or ICI 182,780. In untreated MCF-7 cells, clusterin is synthesized as 50–53 kDa protein that is initially glycosylated in the ER, presumably by oligosaccharide transferase (OST) during cotranslational transport through the translocon.[13] The nascent glycoprotein is modified by the ER-resident glucosidase and mannosidase I, folded with the assistance of calnexin and ERp57, and after disulfide bond formation the protein is transported to the Golgi apparatus.[47] Further processing of the oligosaccharide chains in the Golgi by modifying enzymes including mann II and Gal T, and cleavage of the proprotein produces the mature 70–80 kDa glycoprotein containing distinct disulfide-linked α and β chains.[48]

The biosynthesis of clusterin is disrupted at several levels after the induction of apoptosis by TNFα or ICI 182,780. First, while the nascent protein is translocated from the ER to the Golgi, the protein is not modified by proteolysis to produce the α and β chains. Secondly, after treatment of MCF-7 cells with either compound, clusterin accumulates in the ER and Golgi, and subsequently in the nucleus. The nuclear isoform is disulfide linked, indicating that the protein is still cotranslationally translocated to the ER after treatment. However, the protein is not glycosylated, implying that either the nascent protein is not initially glycosylated by OST, or that the glycoprotein is deglycosylated prior to retrograde transport to the ER and nucleus. While it is not possible to distinguish between these two processes based on the present data, the former process is more likely. The initial glycosylation events are mediated by OST during translocation into the lumen of the ER, which is only active if DAD-1 (defender against death) is associated with the OST complex.[49–52] Thus, in the absence of DAD-1, clusterin (and other glycoproteins) will not be appropriately cotranslationally modified. In the absence of DAD-1, the protein will be translocated in the ER and the nonglycosylated protein will still be correctly folded (through interaction with calreticulin and Grp78), disulfide linked (by peptide disulfide isomerase) in the ER, and translocated to the Golgi. However, the nonglycosylated isoform of clusterin is not cleaved in the Golgi by the endopeptidase, suggesting that the proteolytic processing is restricted to the fully glycosylated isoform. The mechanism of DAD-1 inactivation is not well understood. There are no obvious phosphorylation or caspase cleavage sites in the protein that might provide an explanation for the inactivation of DAD-1 after the initiation of apoptosis via either the intrinsic or extrinsic cell death pathways.[49] This raises the possibility that the activity of the OST complex may be mediated by transcriptional regulation of DAD-1 levels.

Since the uncleaved 50–53 kDa isoform of clusterin is seen in the Golgi after the induction of apoptosis, the subsequent accumulation of clusterin in the ER appears to be because of the failure of the endopeptidase to cleave the protein, leading to the retrograde transport of the uncleaved, nonglycosylated protein from the Golgi back to the ER. This process is distinct from that induced by BFA, swainsonine or tunicamycin, since these drugs also block the anterograde transport of clusterin from the ER to the Golgi.[53–55] In this respect, the changes in the glycosylation of clusterin are reflected in the changes in the synthesis and glycosylation pattern of mann II and Gal T, two proteins that are resident in the *cis*- and medial Golgi respectively.[39,56] The levels and degree of glycosylation (as assessed by the changes in mobility on Western analysis) indicate that both TNFα and ICI 182,780 have relatively pleotropic effects on the glycosylation processes that are not only restricted to changes in the glycosylation of secreted proteins but also affects modifying enzymes resident in the Golgi and probably ER, implying that the glycosylation pattern of many, if not all, glycoproteins will be altered significantly.

The presence of clusterin in the nucleus has been noted previously under several other pathophysiological conditions, including human hepatoma HepG2 and mink lung epithelial CCL64 cells treated with TGFβ,[57] Shionogi mouse mammary carcinomas after repeated hormone ablation[58] and in MCF-7 cells after irradiation.[59] In these previous studies, the nature of the nuclear isoform was not determined, although it has been suggested that the initiation of translation from a second methionine present in the human clusterin sequence ($\alpha^1$2M)

on free ribosomes would produce a protein lacking the signal sequence which should then be translocated to the nucleus. This is unlikely in our view since this particular amino-acid residue is not conserved in rodent species, and, as this present study documents, the isoform that accumulates in the nucleus is a disulfide-linked protein. Furthermore, this very unusual translocation to the nucleus of the disulfide-linked, nonglycosylated isoform of clusterin does not appear to require the NLS. Since mutation of the NLS does not alter the accumulation of nuclear clusterin, this suggests that the nuclear accumulation of clusterin does not require the interaction of the protein with the nuclear import system from the cytoplasm. This also suggests that the integrity of the Golgi/ER endomebrane complex is not compromised and that there is a mechanism that permits the translocation of the disulfide-linked clusterin across the inner nuclear membrane. It is interesting to note that the ER and inner nuclear membrane are contiguous, and although the mechanism does not appear to involve the formation of new pores, the process is selective since overload of the ER after BFA, swainsonine or tunicamycin does not induce nuclear accumulation of clusterin. These changes in clusterin biogenesis during apoptosis appear to be contemporaneous with ER overload, but precede the proteolytic degradation of the nuclear lamins that marks the initiation of nuclear fragmentation (O'Sullivan, unpublished observations), suggesting that the translocation is not a very late event that only occurs after the integrity of the nuclear membrane has been lost. It is also possible, but unlikely in our view, that the accumulation of nonglycosylated, disulfide-linked isoform in the nucleus is a by-product of the retention of the protein in the ER membrane because of the failure of the signal peptidase to cleave the signal sequence from the nascent protein. While this might lead to the appearance of clusterin in the inner nuclear membrane since the ER and nuclear membranes are contiguous, it does not adequately explain the apparent localization of the protein throughout the nucleoplasm.

Clusterin is localized to the nuclei of cells undergoing DNA fragmentation. This may either be because of a generalized breakdown of the nuclei, or because of a specific pathway. As shown by FFE and confocal microscopy after treatment with TNFα or ICI 182,780, clusterin accumulates in the ER and nucleus of dying MCF-7 cells. Analysis of multiple fields on confocal microscopy has established that nuclear clusterin accumulation invariably occurs in cells that are TUNEL positive and appears to occur slightly after DNA fragmentation. This suggests that the nuclear accumulation of clusterin occurs via a regulated mechanism, but also makes it unlikely that nuclear clusterin is involved in the activation of the apoptotic endonuclease(s), at least in MCF-7 cells. In a yeast two-hybrid screen clusterin, identified as XIP-8, a radiation-induced protein,[59] has been shown to bind Ku 70, one of the regulatory components of the DNA-dependent protein kinase responsible for activating double-stranded DNA repair processes.[60] While these studies utilized a construct of human clusterin that will not produce a disulfide-linked protein (since the signal sequence was removed in the construct and translation consequently initiated on α^{12}M), they suggest that the nuclear isoform of clusterin may regulate DNA repair. Thus, in the context of cell death initiated through the extrinsic pathway by TNFα, or the estrogen receptor-mediated pathway by ICI 182,780, it is likely that nuclear clusterin leads to the sequestration of Ku70 away from DNA repair processes, leading to the inhibition of DNA repair at a time that endonucleases are responsible for the orderly destruction of the genome. Within this framework, clusterin can be regarded as a sentinel protein, whose translocation to the nucleus ensures that appropriate DNA cleavage ensues without unwanted DNA repair that might lead to substantial genomic instability were the cell to survive.

In summary, the data presented in this report demonstrate that alterations in the biogenesis of clusterin are induced during cell death, apparently as a result of changes in glycosylation and proteolytic processing. These alterations lead to changes in the subcellular location and, almost certainly, in the function of the protein. These observations may reconcile many of the differences in the literature regarding the role of clusterin during apoptosis. If the current observations are extended to other cell types undergoing apoptosis, it would suggest that the nuclear isoform clusterin is neither anti- nor proapoptotic, since it regulates DNA repair processes to prevent inappropriate DNA repair and genomic instability. It is interesting to note that these changes in clusterin function, while having profound effects on the cell, do not involve mutations in the gene, but rather represent an example of the major epigenetic effects that are not detectable using genetic methodologies such as positional cloning or genomic analysis.

Materials and Methods

Determination of cell growth

MCF-7, an estrogen receptor-positive and hormone-sensitive human breast carcinoma cell line was obtained from ATCC and maintained as a monolayer culture in α-MEM with Earle's salts, L-glutamine with HEPES, $NaHCO_3$, glucose, supplemented with 5% fetal bovine serum (FBS) and streptomycin sulfate/penicillin. Crystal violet assays were used to measure cell growth. MCF-7 cells were plated at 2×10^4 cells per well in 24-well dishes, grown overnight in serum containing α-MEM medium and transferred to phenol red-free medium supplemented with 5% charcoal-stripped serum (CSS) and 10 nM 17β-estradiol prior to treatment. At various times after treatment, the cells were fixed in 1% glutaraldehyde for 15 min at room temperature. Crystal violet solution (0.1%) was added and incubated for 30 min at room temperature. Excess dye was discarded and 0.2% Triton X-100 was added to each well. Control cultures were treated with PBS, ethanol or methanol, all of which were used as vehicle controls where appropriate. Absorbances were measured at A_{590} using a Victor^2 microplate reader (Wallac, Gaithersburg, MD, USA).

Statistical evaluation

Statistical analyses were performed using GraphPad Instat (GraphPad Software, San Diego, CA, USA). Data are expressed as mean $\pm$ S.E.M. One-way analysis of variance was used to assess statistical significance between means. Differences between means were considered significant if *P*-values less than 0.05 were obtained using the Bonferroni method.

Cell growth conditions for FFE

FFE is a gentle, fast and efficient method for the preparative separation of cell organelles and membrane systems.[61–63] MCF-7 cells were seeded at 1×10^7/ml in roller bottles, incubated in α-MEM supplemented with 5% FBS and streptomycin sulfate/penicillin for 48 h prior to treatment and transferred to phenol red-free medium supplemented with 5% CSS and 10 nM 17β-estradiol 24 h prior to treatment and harvested at fixed times of treatment. For individual experiments, MCF-7 cells were treated with either 1 ng/ml TNFα, or 10 μM ICI 182,780 or 5 μg/ml BFA, prior to harvest at times at which the majority of cells were determined to be in the early stages of the apoptotic process prior to the formation of apoptotic bodies (24 h after treatment for TNFα, or ICI 182,780 treatment and 6 h for BFA experiments). Control cultures were treated with ethanol or methanol (as vehicle controls).

Cell homogenization using a ball-bearing cell cracker

All manipulations and solutions were at 0–4°C unless otherwise indicated. Monolayers were rinsed twice with cold PBS and trypsinized. The viable cells were resuspended in $TEAs_{250}$ (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.4) at a density of 3×10^7/ml, placed on ice for 5 min and homogenized with a ball-bearing cell cracker (custom made by H & Y Enterprises, Redwood City, CA, USA). The optimum clearance and the number of passages needed to obtain 80% cell breakage while leaving greater than 90% of the nuclei intact were determined empirically using trypan blue dye exclusion assay. For the untreated MCF-7 cells, cells were passed through the homogenizer five times using a clearance of 27.9 μm, whereas treated MCF-7 cells passed through the homogenizer five times with a clearance of 22.8 μm. The difference in clearance between untreated and treated cells is attributable to the slight but significant decrease in the diameter of the cells after treatment with TNFα, ICI 182,780 or BFA (O'Sullivan, unpublished observations). The homogenates were incubated at room temperature for 10 min and centrifuged for 10 min at 250 $\times$ g to derive the nuclear fraction and the postnuclear supernatant (PNS).

Nuclear isolation

The nuclear fraction was washed twice in PBS and resuspended in 1 ml buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.25 mM PMSF, 0.25 mM benzaminide 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1% β-mercaptoethanol, pH 7.9), centrifuged at 10 000 $\times$ *g* for 10 min and resuspended in buffer A plus 1% NP-40 for 10 min with frequent vortexing. The integrity of the nuclear membranes was verified by light microscopy. The nuclei were layered onto a sucrose cushion (15 mM HEPES, 60 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 0.876 M sucrose, pH 7.9) and pelleted at 10 000 $\times$ *g* for 10 min. The nuclear pellet was resuspended in buffer C (20 mM HEPES, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 25% glycerol, 0.25 mM PMSF, 0.25 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.9) by gentle mixing, incubated on ice for 30 min and pelleted at 10 000 $\times$ g for 5 min. For high salt extraction of nuclear proteins, pellets were incubated for 20-min on ice in 20 mM HEPES, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 25% glycerol, 0.25 mM PMSF, 0.25 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 420 mM NaCl, pH 7.9, centrifuged at 10 000 $\times$ *g* for 3 min and the resulting supernatants stored at -80°C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL,USA).

Free flow electrophoresis (FFE)

The PNS prepared from the cell cracker was made 1.4 M sucrose by the addition of 1.04 vol 2.5 M sucrose and overlaid with 1.25, 0.25 M sucrose and $TEAs_{250}$. The gradient was centrifuged for 1.5 h at 35 000 rpm in a Beckman SW-41Ti rotor. The organelles were collected as low-density membranes (LDM) at the 0.25/1.25 sucrose interface. FFE was performed using an Octopus FFE instrument (Weber GmBH, Germany) at 6°C using a constant current of 120 mA and a field of 1200 V. The LDM samples were fractionated at 50 ml/min and collected into a 90-sample fraction collector. The approximate transit time in the separating chamber was 5 min. Individual runs took 30–60 min depending on the volume of the initial sample.

Marker enzyme assays

The localization of individual membrane fractions and the purity of the fractions was determined using established marker enzymes for different organelles: β-hexosaminidase for lysosomes, mann II for the medial Golgi, alkaline phosphodiesterase for the plasma membrane and glucose-6-phosphatase for the ER, essentially as previously described.[63–65]

Cytosolic protein isolation

Cells were seeded at 1×10^6/ml in T75 flasks, grown overnight in serum-containing culture media and transferred to phenol red-free media supplemented with 5% CSS and 10 nM 17β-estradiol. At various times after treatment, monolayers were trypsinized and the cell suspension was centrifuged at 2000 rpm for 7 min. The cell pellet was then homogenized in RIPA buffer (10 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium flouride, 10 mM sodium vanadate, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 2 mM Pefabloc, and 1 mM benzamidine, pH 7.5) to isolate cytosolic protein. The homogenate was placed on ice for 20 min, sonicated three times for 20 s on ice, centrifuged at 10 000 $\times$ *g* for 15 min and the supernatants stored at -20°C until use.

Immunofluorescence

Cells were seeded at 2×10^4 cells/ml in slide chambers (Nalgene), grown overnight in serum-containing media and transferred to phenol red-free media supplemented with 5% CSS and 10 nM 17β-estradiol prior to treatment. At various times after treatment, the monolayers were washed twice for 5 min with PBS and fixed with 3.7% formaldehyde for 10 min at room temperature. The cells were permeabilized with 100% cold methanol at -20°C for 6 min, washed and incubated at room temperature in PBS containing 1% BSA for 30 min and then incubated with the appropriate primary antibody for 1 h with gentle agitation at room temperature, followed by two 10 min washes with PBS containing 1% BSA. Slides were incubated with secondary antibody for 1 h at room temperature, followed by three 10 min washes with PBS containing 1% BSA, mounted with Poly-aqua and processed for confocal laser scanning microscopy using a Nikon microscope (Nikon, Diaphot 200) equipped with Biorad scanning software.

TUNEL analysis and clusterin colocalization

Cells were seeded at 8000 cells per chamber in CC2-coated chamber slides (Nalge Nunc International, Naperville, IL, USA) and grown as

described above. Cells were treated with 1–10 μM ICI 182,780 or 1–10 ng/ml TNFα for 72 h in DMEM: Hams F12 plus 5% charcoal-stripped serum and 10 nM estradiol. Following treatment, the monolayers were washed with PBS, fixed with 3.7% formaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 6 min at -20°C. TUNEL analysis was performed according to the *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN, USA) using terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides. Positive controls for TUNEL included MCF-7 cells treated with 1 μg/ml DNase1 to induce multiple DNA strand breaks. Slides were washed three times for 5 min with PBS prior to mounting. For colocalization studies with clusterin, after the final wash, the slides were incubated with 1/10 dilution of 6E9, the β-chain-specific monoclonal anti-clusterin antibody for 2 h at room temperature followed by incubation with 1/200 dilution of Cy3-labeled anti-mouse IgG for 1 h at 37°C in a humidity chamber, and washed three times for 5 min with PBS. Slides were mounted with Polyaqua and processed for confocal laser scanning microscopy using a Nikon microscope (Nikon, Diaphot 200) equipped with Biorad MRC 1024 scanning software. Z-series images were acquired using LaserSharp 2000 at a magnification of $\times$ 60 by the double label method (FITC 488 nm, Cy3 568 nm). Images were projected using Confocal Assistant 4.0 and merged using Adobe Photoshop 5.0.

Western blot analysis

SDS polyacrylamide gels (12.5%) were run at 100 V for 90 min under reducing conditions. Gels were electroblotted at 200 mA for 2 h to nitrocellulose membranes and blocked for 1 h with 1% heat-denatured casein. The membranes were probed with the appropriate primary and secondary antibodies. Immunoreactive bands were visualized by chemiluminescence using the Pierce kit (Pierce, Rockford, IL, USA) and exposed to X-Omat AR film (Eastman Kodak Co, Rochester, NY).

Deglycosylation of N-linked oligosaccharides

Purified nuclear proteins were denatured in 0.5% SDS 1% β-mercaptoethanol for 10 min at 100°C and incubated overnight at 37°C with 0.5 M sodium phosphate buffer and 10% NP-40, pH 7.5 and 1000 U of PNGase F (NEB) in a total volume of 30 μl. Mock controls were treated as above, without addition of PNGase F, and either incubated overnight or frozen immediately at -20°C. Samples were analyzed by SDS-PAGE and clusterin isoforms were visualized by Western analysis using the monoclonal anticlusterin antibody, 6E9 (1/10), and developed using enhanced super signal chemiluminescence kit (Pierce) and exposed to X-Omat AR film (Kodak) at room temperature for 10 min.

Mutagenesis

Mutagenesis was performed using the Site-directed Mutagenesis Kit using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). Briefly, complementary primer pairs to the template were designed with the desired sequence to be mutated present in the middle of the primer sequence. To mutate the NLS, PCR of the mutated sequence was performed using the following cycle conditions: forward (5′-AGAA-GAAGCCGAGAAGGAGAAAGAGGATGC-3′) and reverse (5′-GCATCCTCTTTCTCCTTCTCGGCTTCTTCT-3′) primers, containing the sequences to be mutated (underlined), were used to PCR amplify the mutated DNA using the following conditions: hot start 30 s at 95°C, 30 s at 95°C, 1 min at 60°C, 9 min at 68°C, for 30 cycles. The PCR-amplified DNA was restriction digested with Dpn1 and transformed into XLI-Blu highly competent cells. Transformants were screened and sequenced. The resulting mutated cDNA was subcloned into the pcDNA 6 vector containing a myc tag (Invitrogen), sequenced to confirm location of the mutation and stably transected into MCF-7 cells.

Generation of stable transfectants

MCF-7 cells were seeded at 5×10^5cells/ml in a T25 flask and grown overnight. At 70% confluency, cells were transfected with the myc tagged mutated clusterin constructs or the empty vector using lipofectamine (Gibco BRL) in serum-free media for approximately 18 h. The transfection serum free media were removed and cells were incubated in serum-containing media for 24 h and subsequently selected using 10 μg/ml blasticidin for approximately 10 days. Surviving cells were tested for expression by Western blot analysis using an antibody to the myc tag (Invitrogen).

Antibodies and reagents

Mouse anti-human clusterin antibody (6E9) that recognizes the β chain of clusterin was developed in our laboratory.[37] The antibody specific for manno II was generously provided by Dr. Kelley Moreman (University of Georgia, Athens, GA, USA) and anti-Gal T by Dr. Eric Berger (University of Zurich, Zurich, Switzerland). BFA was purchased from Sigma and stored as stock solutions in methanol. TNFα was purchased from Sigma and ICI 182,780 was provided by Zeneca plc (Macclesfield, UK).

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