Clusterin Has Chaperone-like Activity Similar to That of Small Heat Shock Proteins*

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Clusterin is a highly conserved protein which is expressed at increased levels by many cell types in response to a broad variety of stress conditions. A genuine physiological function for clusterin has not yet been established. The results presented here demonstrate for the first time that clusterin has chaperone-like activity. At physiological concentrations, clusterin potently protected glutathione S-transferase and catalase from heat-induced precipitation and α-lactalbumin and bovine serum albumin from precipitation induced by reduction with dithiothreitol. Enzyme-linked immunosorbent assay data showed that clusterin bound preferentially to heat-stressed glutathione S-transferase and to dithiothreitol-treated bovine serum albumin and α-lactalbumin. Size exclusion chromatography and SDS-polyacrylamide gel electrophoresis analyses showed that clusterin formed high molecular weight complexes (HMW) with all four proteins tested. Small heat shock proteins (sHSP) also act in this way to prevent protein precipitation and protect cells from heat and other stresses. The stoichiometric subunit molar ratios of clusterin:stressed protein during formation of HMW complexes (which for the four proteins tested ranged from 1.0:1.3 to 1.0:11) is less than the reported ratios for sHSP-mediated formation of HMW complexes (1.0:1.0 or greater), indicating that clusterin is a very efficient chaperone. Our results suggest that clusterin may play a sHSP-like role in cytoprotection.

Clusterin was first described in 1983 as a major secretory glycoprotein produced by ram Sertoli cells (1). It is a 75–80-kDa disulfide-linked heterodimeric protein with about 30% of the mass of the molecule comprised of N-linked carbohydrate which is branched, complex, and rich in sialic acid (2). Clusterin is transcribed from a single structural gene as a full-length mRNA of 1.6–2.1 kilobases (depending on the length of the polyA tail) and the translated product is internally cleaved to produce the two subunits prior to secretion from the cell. Clusterin is translated with a typical hydrophobic signal peptide, 21 amino acids in length, which is proteolytically removed during translocation of the protein to the endoplasmic reticulum lumen (3). Although clusterin is usually secreted from cells, it has been reported that treatment of HepG2 and CCL64 cells with transforming growth factor β induces translation of a truncated form of clusterin (lacking the signal peptide) which remains intracellular (4).

One of the most striking things about clusterin is the breadth of its biological distribution. In animal tissues clusterin mRNA is near ubiquitous, with reports describing its occurrence in locales as diverse as the rat prostate gland, the velvet antler from red deer, and quail neuroretinal cells. Across this broad species range clusterin maintains a remarkably high level of sequence homology, comparisons between mammalian species typically being in the range of 70–80% (3). The wide distribution and sequence conservation of clusterin suggest that the protein performs a function of fundamental biological importance. Furthermore, increased clusterin expression is found in a variety of disease states and experimental models of pathological stress (5), including heat shock (6, 7). Therefore, it is reasonable to suppose that the biological function(s) of clusterin may have immediate relevance to disease states and stress conditions.

Many binding interactions between clusterin and other biological molecules have been described. We have shown that clusterin binds to immunoglobulins (8), lipids (9), heparin (10), glutathione S-transferase (GST),1,2 and the surfaces of pathogenic isolates of Staphylococcus aureus (11). Others have reported that clusterin also binds to terminal complement components C7, C8, and C9 (12), apolipoprotein A-I (13), paraoxonase (14), amyloid β peptide (15), gp330 (an endocytic receptor related to the low density lipoprotein receptor (16)), and a protein secreted by Streptococcus pyogenes (17). Each description of a new clusterin-binding interaction has led to a proposal that clusterin is involved with the specific biological function of the binding partner. Consequently, functions proposed for clusterin are as diverse as its reported binding partners. Thus, it has been suggested that clusterin acts as a phagocyte recruitment signal (18), promotes cell aggregation (19), protects cells from complement attack (20, 21), regulates apoptosis (18, 22), protects cells at fluid-tissue interfaces from harsh conditions (23), re-models membranes (24), and transports lipids (25). None of these has been established as a genuine physiological function.

Sequence analysis predicts that clusterin has three putative amphipathic α-helical regions, a type of secondary structure thought to be important in mediating interactions with hydrophobic molecules (26, 27). Many of the reported biological ligands of clusterin are significantly hydrophobic. Therefore, we

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1 The abbreviations used are: GST, glutathione S-transferase; sHSP, small heat shock protein; HMW, high molecular weight; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SMR, subunit molar ratio; mAb, monoclonal antibody.

2 S. B. Easterbrook-Smith, unpublished results.
reasoned that interactions of these molecules with clusterin may reflect a general propensity of clusterin to bind to hydrophobic regions of molecules, regardless of their biological function. In this context, the recent demonstration that a highly conserved 14-base pair elements, shared by all vertebrate clusterin proximal promoters, specifically recognizes the heat shock factor 1 transcription factor is of significant interest. This 14-base pair element was shown to be capable of mediating heat shock-induced transcription (28). These authors suggested that this heat-responsive element provides an explanation for the high sensitivity of clusterin expression to environmental changes and proposed that clusterin may function as an extracellular heat shock protein (28).

We hypothesized that the function of clusterin expressed during cellular stress, like the small heat shock proteins (sHSPs), may be to act in a chaperone-like manner and bind to hydrophobic regions of partly unfolded, stressed proteins, thereby “solubilizing” them and protecting cells from the cytotoxic consequences of protein precipitation. As a first step toward testing this hypothesis, we examined the ability of clusterin to protect a variety of proteins from stress-induced protein precipitation. To do so, we prepared a solubilized high molecular weight (HMW) complex.

**Experimental Procedures**

Reagents—Bovine serum albumin (BSA), catalase, 1-chloro-2,4-dinitrobenzene, glutathione (GSH), H$_2$O$_2$, iodoacetamide, and α-lactalbumin were all obtained from Sigma. Dithiothreitol (DTT) was obtained from Boehringer-Mannheim (Sydney, Australia). All other chemicals were obtained from Ajax (Sydney, Australia). GST from Schistosoma japonicum was prepared by thrombin cleavage of recombinant Jun leucine zipper-GST fusion protein and purified by GSH-affinity chromatography as described (29). Clusterin was purified from human serum by immunoaffinity chromatography as described previously (8).

Protein Precipitation Assays—Individual solutions of clusterin (10–200 μg/ml), catalase (200 μg/ml), or GST (200 μg/ml), or mixtures of clusterin with catalase or GST at the same final concentrations, were prepared in 0.7 ml of 50 mM sodium phosphate, pH 7.0, and heated at 60 °C. The light scattering of the solution at 360 nm was measured every 30 s for a total of 25 min in an automated 7-channel diode array spectrophotometer (Hewlett-Packard GMBH, Germany). Individual solutions of clusterin (10–200 μg/ml), α-lactalbumin (2.5 mg/ml), or BSA (2.5 mg/ml), or mixtures of clusterin with α-lactalbumin or BSA at the same final concentrations, were prepared in 0.3 ml of 50 mM sodium phosphate containing 0.1 mM NaCl and incubated at 37 °C with or without 20 mM DTT. During this period absorbance readings at 360 nm were acquired every 5 min for a total of 5 h in a Spectramax 250 plate reader (Molecular Devices, Sunnyvale, CA).

Enzyme Assays—Catalase was prepared at a concentration of 200 μg/ml in 0.1 M sodium phosphate (pH 7.0) with or without clusterin (100 μg/ml). Mixtures were heated at 37 or 55 °C for 30 min. 1.0 μl of the catalase/clusterin mixture was incubated with 1.0 ml of H$_2$O$_2$ substrate solution (0.12% (w/v) H$_2$O$_2$ in 50 mM Na$_2$HPO$_4$, pH 7.0) at 37 °C for 5 min before the reaction was stopped with 150 μl of 4 M NaOH. The absorbance of H$_2$O$_2$ was measured at 250 nm on a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Enzyme activity was measured as a decrease in absorbance. GST was prepared at a concentration of 200 μg/ml in 50 mM Na$_2$HPO$_4$, pH 7.0, with or without clusterin (100 μg/ml). Mixtures were heated at 37 or 50 °C for 30 min. GST was then diluted into substrate solution (1 mM GSH, 2 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate, pH 7.4) to a final concentration of 4.5 μg/ml and incubated at 37 °C for 5 min before measuring the absorbance at 350 nm. Enzyme activity was measured as an increase in absorbance, corresponding to the appearance of 1-S-glutathionyl-2,4-dinitrobenzene.

ELISA—GST and catalase, at 20 μg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, pH 7.4), were adsorbed onto ELISA trays (Disposable Products, Adelaide, Australia) for 1 h at 37 °C. In some cases, after the coating step, the trays were heated to 60 °C for 30 min to stress the adsorbed proteins. BSA and α-lactalbumin were applied to ELISA trays in PBS at a concentration of 1.0 mg/ml, with or without 20 mM DTT, and incubated overnight at 37 °C. Plates were then blocked with 1% (w/v) heat-denatured casein prepared in PBS, pH 7.4 (i.e., 1% heat-denatured casein). Plate-bound DTT-treated proteins were then incubated with 5 mM iodoacetamide for 1 h at 37 °C, in order to exclude the possibility of subsequent formation of disulfide bonds between clusterin and the other proteins. Clusterin, initially at 10 μg/ml, was then serially diluted in binary steps across the ELISA tray, using
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Enzyme activity of (A) GST and (B) catalase following incubation at either 37 or 50/55 °C. Solutions containing the specified enzyme ± clusterin were heated for 30 min at the temperature indicated before measuring enzyme activity. In each case, the table below the x axis indicates the conditions applied (indicated by an x). GST activity was measured as an increase in absorbance at 350 nm following the addition of substrate, representing the appearance of 1-glutathionyl-2,4-dinitrobenzene. Catalase activity was measured as a decrease in absorbance at 250 nm, representing the disappearance of H₂O₂ following the addition of substrate, representing the appearance of 1-glutathionyl-2,4-dinitrobenzene. Catalase activity was measured as a decrease in absorbance at 250 nm, representing the disappearance of H₂O₂. Further description of these activity assays is provided under standard methods by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). A Hoefer "Tall Mighty Small" apparatus (Australian Chromatography Co., Sydney) was used at a constant current of 30 mA. Gels were stained with standard Coomassie Blue reagent.

**RESULTS**

Clusterin Protects Proteins from Stress-induced Precipitation but Does Not Protect Enzymes from Heat-induced Loss of Function—Heating GST or catalase at 60 °C produced extensive protein precipitation within 30 min, shown by an increase in absorbance at 360 nm (Fig. 1, A and B). Likewise, reduction of BSA or α-lactalbumin with DTT resulted in extensive protein precipitation within 4 h (Fig. 1, C and D). In contrast, clusterin did not precipitate when heated at 60 °C for 30 min or when treated with DTT for 5 h (data not shown). When co-incubated with any of the proteins subjected to heat or DTT-mediated reduction, clusterin potentely inhibited protein precipitation (Fig. 1, A-D). Since many chaperones (and also clusterin) exist in solution as aggregates of an ill-defined number of monomers, a convention that has been adopted when dealing with the interactions between chaperones and other proteins is to define stoichiometry in relation to the individual subunits of the chaperone and the protein with which it interacts. For these calculations we have assumed a molecular mass for intact clusterin of 80 kDa and a subunit mass of 40 kDa. An approximate subunit molar ratio (SMR) of clusterin:GST of 1.0:3.2 was the minimum required to virtually abolish reduction-induced precipitation (Fig. 1A). Corresponding SMRs for the stabilizing effects of clusterin on the other proteins tested were 1.0:1.3 (catalase), 1.0:2.3 (BSA), and 1.0:1.1 (α-lactalbumin) (Fig. 1, B, C, and D, respectively). In contrast, even at corresponding SMRs as high as 1.4:1.0, a control protein (ovalbumin) had only a small effect on the stress-induced precipitation of any of the proteins tested (data not shown).

To investigate whether clusterin is also capable of protecting enzymes from stress-induced loss of function, we tested the enzyme activity of GST and catalase before and after exposure to 60 °C for 30 min in the presence or absence of clusterin. The presence of clusterin (at sufficient concentration to provide protection against precipitation; Fig. 1, A and B) had no effect on the loss of enzyme activity in either case (Fig. 2). Overall, these results indicate that clusterin has potent heat- and reduction-stable chaperone-like properties which are capable of protecting stressed proteins from precipitation but which cannot protect GST or catalase from heat-induced loss of enzyme activity.

Clusterin Binds Preferentially to Stressed Proteins—In ELISA, clusterin did not bind significantly to any of the non-stressed proteins tested, with the exception of GST (Fig. 3). Binding of clusterin to unstressed GST had been previously noted. Clusterin showed significantly increased binding to heat-stressed GST (Fig. 3A) and significant binding to DTT-treated BSA and α-lactalbumin (Fig. 3, C and D). Thus, our ELISA results indicate that, comparing untreated versus stressed GST, BSA, and α-lactalbumin, clusterin binds prefer-
resulted in partial dissociation of large clusterin aggregates to form monomers (80 kDa species) or lower order clusterin oligomers but did not appear to cause substantial dissociation of the α and β chains.

In all cases, when clusterin was added to proteins undergoing heat- or DTT-mediated stress, analysis of the mixtures by size exclusion chromatography indicated pronounced formation of HMW species (Fig. 4, A-D, indicated by solid arrows). The HMW species were not detected in analyses of clusterin alone or any of the individual proteins, whether these molecules were stressed or not. Nor were they detected in mixtures of clusterin and any of the other proteins in the absence of experimental stress (Fig. 4, A-D). In each case, SDS-PAGE analysis of the HMW species in the exclusion volume peaks indicated the presence of both clusterin and the stressed protein under test (Fig. 5). Thus, these results indicate that clusterin, like the sHSPs, binds to stressed proteins to form HMW complexes. The only other possible explanation for these results is that, in each of four different cases, when clusterin and the “target” protein are present together in solution, but not when either protein is present alone in solution, clusterin and the target protein each undergo stress-induced homo-oligomerization. This latter explanation seems implausible, particularly in light of the demonstrations that (i) clusterin inhibits stress-induced protein precipitation (Fig. 1) and (ii) clusterin binds to stressed proteins in ELISA (Fig. 3). We also analyzed the protein mixtures produced for size exclusion chromatography by native gel electrophoresis (at pH 7.0). When mixtures of clusterin and either GST or BSA were exposed to heat or DTT, respectively, but not in the absence of experimental stress, these analyses demonstrated the formation of a new stress-induced species of unique electrophoretic mobility (data not shown). Similar analyses of
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It was previously shown that clusterin reduced the aggregation of a synthetic amyloid β-peptide in aqueous solution (15). However, effects of clusterin on the aggregation of stressed proteins have not been reported before. The results presented here demonstrate for the first time that clusterin has chaperone-like activity. Clusterin potently protects GST and catalase from heat-induced precipitation and α-lactalbumin and BSA from precipitation induced by reduction. Preliminary experiments indicate that clusterin also protects IgG and ovotransferrin from heat-induced precipitation.3 Thus, clusterin has the ability to protect many different proteins from stress-induced precipitation. However, in the case of two enzymes tested, GST and catalase, clusterin was unable to protect against heat-induced loss of activity. The sHSPs, HSP25, and α-crystallin, also protect catalase from thermally induced precipitation but, interestingly, α-crystallin is capable of protecting against enzyme inactivation while HSP25 is not (34). We have not yet determined if clusterin has any effects on the recovery of enzyme activity following a heat shock.

When tested in ELISA for binding to the native form of the four proteins tested in this study, clusterin bound only to GST. However, clusterin bound more strongly to heat-treated than to untreated GST and also bound significantly to reduced α-lactalbumin and BSA. Thus, in the case of GST, α-lactalbumin, and BSA, these results are compatible with a model in which stress caused partial unfolding of these proteins to expose binding site(s) for clusterin. For all four proteins examined, when clusterin was present in solutions of stressed proteins and these mixtures analyzed by size exclusion chromatography, a HMW fraction (of mass greater than the exclusion limit of the column, 1.5 × 10^6 Da) was detected. In each case, SDS-PAGE analysis of this HMW fraction indicated that it was comprised of both clusterin and the target protein. It was concluded that clusterin formed HMW complexes (of mass greater than 1.5 × 10^6 Da) with each of the stressed proteins tested. This conclusion was further supported by native gel electrophoretic analysis of mixtures of clusterin and stressed proteins (data not shown).

The results discussed above clearly indicate that clusterin binds to heat-stressed catalase to form a HMW complex. This was further confirmed by demonstrating by SDS-PAGE analysis that the G7 (anti-clusterin) mAb immunoprecipitates a complex of clusterin and catalase from heat-stressed mixtures of the two proteins (data not shown). Yet, clusterin failed to bind to heat-stressed catalase in ELISA (Fig. 3B). One possible explanation for this apparent discrepancy is that steric constraints imposed by the solid phase adsorption of catalase may have interfered with binding interactions between the two proteins in this assay format. This hypothesis is consistent with the demonstration that solution phase, heat-stressed catalase inhibited the binding of clusterin to solid phase-adsorbed, heat-stressed GST (Fig. 3B, inset). Presumably, this resulted from clusterin complexing with catalase in the solution-phase, leading to less clusterin available for interaction with the solid-phase GST.

No crystal structure is available for clusterin and very lim-

3 D. Humphreys and S. Poon, unpublished results.
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Predictions based on analysis of the sequence of human clusterin cDNA. The hydrophobicity index (MacVector v4.14, Hopp-Woods scale) is plotted as a histogram display. Predicted regions of amphipathic α-helical regions, secondary structures thought to be important in interactions with hydrophobic molecules (26). In addition, sequence analysis predicts a number of short regions of hydrophobicity outside those defined by the putative amphipathic α-helical regions (Fig. 6). Since clusterin is known to bind to a variety of native proteins with hydrophobic domains, it is likely that clusterin exerts its chaperone-like activity by binding to exposed hydrophobic regions of stressed proteins. As appears likely for clusterin, sHSPs are known to bind to exposed hydrophobic regions on stressed proteins to form HMW complexes (35). The formation of these complexes “solubilizes” stressed proteins, preventing their precipitation. It is also known that, generally, when acting alone, sHSPs are unable to protect enzymes from heat-induced loss of function (33, 36). These characteristics appear to be shared with clusterin.

The low SMRs of interaction between clusterin and the four proteins tested (ranging from 1.0:1.3 to 1.0:11) indicate that clusterin is a very efficient chaperone. In comparison, the sHSPs are less efficient in their interactions with stressed proteins; the available data suggest that sHSPs bind stressed proteins at a SMR of one or more subunits of sHSP to one partially folded protein subunit (i.e. at best, SMR = 1.0:1.0 (37, 38)). Comparing catalase and GST, the catalase subunit (catalase is a homotetramer of 59.7-kDa subunits) is greater in mass than GST (a 25.5-kDa monomer) by approximately a factor of 2.3. The SMRs corresponding to the minimum amounts of clusterin required to inhibit heat-induced precipitation of catalase and GST were 1.0:1.3 and 1.0:3.2, respectively. Thus on a molar subunit basis, approximately 2.5-fold more clusterin was required to prevent heat-induced precipitation of catalase versus GST, a factor that is very similar to the ratio of their subunit masses (Table I). A similar analysis reveals that the

| Protein(s)        | Subunit molecular mass | Minimum SMR (clusterin:target protein) | Ratio of subunit molecular masses | Ratio of minimum SMRs* |
|-------------------|------------------------|----------------------------------------|----------------------------------|-------------------------|
| Catalase          | 59.7                   | 1.0:1.3                                | 2.3                              | 2.5                     |
| GST               | 25.5                   | 1.0:3.2                                |                                  |                         |
| Catalase versus GST |                       |                                        |                                  |                         |
| BSA               | 69.2                   | 1.0:2.3                                | 4.9                              | 4.8                     |
| BSA versus α-Lactalbumin | 14.1             | 1.0:11                                 |                                  |                         |

* Required to inhibit stress-induced protein precipitation.

Differences in secondary structure and the charge distribution of the polypeptide backbone of the β-catenin C-terminal domain (ΔCTD) and the phosphorylated counterpart were quantified by HPLC (+SDS) with a well characterized, ligand-induced conformational transition as a reference system. The method allowed for a detailed description of the complex interplay between the electrostatic potential and the secondary structure of the β-catenin CTD and its importance in protein function.
subunit molecular mass of BSA is approximately 4.9-fold greater than that of α-lactalbumin and, relative to α-lactalbumin, on a molar basis, BSA requires 4.8 times more clusterin to effect comparable inhibition of reduction-induced precipitation (Table I).

These data suggest that (i) for heat-stressed proteins, the efficiency of the chaperone-like action of clusterin is inversely proportional to the subunit molecular mass of the target protein, and (ii) a similar relationship applies to proteins stressed by reduction. However, there is not a simple uniform relationship (for all the proteins tested, regardless of the type of stress) between the mass of the protein and the efficiency of clusterin chaperone-like action. This is apparent by making comparisons between heat-stressed proteins and those stressed by reduction (see Table I). This suggests that there may be a fundamental difference in the mechanism by which clusterin exerts its chaperone-like action under conditions of stress induced by heat versus reduction. Despite this, regardless of the nature of the stress, the data suggest that steric constraints influence the interaction of stressed proteins with clusterin. When considering proteins stressed by heat, or those stressed by reduction, in each case clusterin was able to interact with and stabilize a greater number of smaller versus larger stressed protein molecules. Interestingly, when a similar comparison is made for the interaction of three reduced proteins (α-lactalbumin, BSA, and ovotransferrin) with the sHSP, α-crystallin, a similar relationship is found (38).

Multiple lines of evidence suggest that clusterin can act to protect cells from environmental stresses. We recently reported that overexpression of clusterin protected murine L929 cells from the cytotoxicity of tumor necrosis factor α (31). It was earlier reported that inhibition of clusterin synthesis by treatment of LNCaP cells with an antisense oligonucleotide enhanced the cytotoxicity of tumor necrosis factor α and that overexpression of clusterin in these cells protected them from tumor necrosis factor α-mediated death (39). Overexpression of sHSPs has also been shown to protect cells from the cytotoxicity of tumor necrosis factor α (40). Furthermore, following the exposure of U937 cells to UV-B irradiation, in situ hybridization detected clusterin mRNA in surviving cells but not in those that had undergone apoptosis (a control mRNA was detected in both live and dead cells) (41). Similarly, increased clusterin mRNA expression was not detected in murine olfactory neurons induced to undergo apoptosis but was detected in surviving glial cells that surrounded the neurons (42). Therefore, it appears that clusterin expression is associated with cell survival.

To summarize, clusterin is a "stress-stable," amphipathic molecule induced in a variety of disease states and instances of cell stress. In vitro, clusterin potently inhibits stress-induced protein precipitation and, in vivo, clusterin expression appears to be associated with cell survival. We speculate that clusterin expression is up-regulated in cells undergoing stress so that clusterin may bind to partly unfolded proteins within and/or outside cells to prevent their precipitation. Similar up-regulated expression of, and an intracellular action by, sHSPs protects cells from stresses and promotes cell viability (43). The concentration of clusterin shown here to protect stressed proteins from precipitation is within the range normally found in some extracellular fluids in mammalian systems (e.g., 50–370 μg/ml in human serum and 2.1–15.0 mg/ml in human seminal fluid (44)), raising the possibility that in these environments clusterin may act as a constitutively expressed chaperone. On the basis of the available evidence, we propose that the chaperone-like activity of clusterin may act to protect cells from heat and other stresses by a mechanism comparable to that of the sHSPs.

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