Evidence That Clusterin Has Discrete Chaperone and Ligand Binding Sites†

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ABSTRACT: Clusterin is the first identified extracellular mammalian chaperone and binds to a wide variety of partly unfolded, stressed proteins.Clusterin also binds to many different unstressed ligands including the cell surface receptor low-density lipoprotein receptor-related protein-2 (LRP-2). It is unknown whether clusterin binds to all of these many ligands via one or more binding sites. Furthermore, the region(s) of clusterin involved in these many binding interactions remain(s) to be identified. As part of an investigation of these issues, we expressed recombinant human clusterin in the yeast *Pichia pastoris*. The resultant protein had variable proteolytic truncations of the C-terminal region of the α-chain and the N-terminal region of the *β*-chain. We compared the chaperone and ligand binding activities of this recombinant product with those of clusterin purified from human serum. We also tested whether the binding of clusterin to ligands could be inhibited by competitive binding with other clusterin ligands or by anti-clusterin monoclonal antibodies. Collectively, our results indicate that (i) clusterin has three independent classes of binding sites for LRP-2, stressed proteins, and unstressed ligands, respectively, and (ii) the binding sites for LRP-2 and stressed proteins are likely to be in parts of the molecule other than the C-terminal region of the α-chain or the N-terminal region of the *β*-chain. It has been suggested that, in vivo, clusterin binds to toxic molecules in the extracellular environment and carries these to cells expressing LRP-2 for uptake and degradation. This hypothesis is supported by our demonstration that clusterin has discrete binding sites for LRP-2 and other (potentially toxic) molecules.

Clusterin is a widely distributed, 70−80 kDa disulfide-linked heterodimeric protein of uncertain function. It was first described in 1983 as a secreted glycoprotein in ram testis fluid that enhanced aggregation ("clustering") of various cells in vitro. Many homologues in other species have subsequently been discovered. Clusterin is encoded by a single gene, and the translated product is (i) internally cleaved to produce its α- and *β*-chains, which are linked by five interchain disulfide bonds, and (ii) extensively glycosylated, such that 30% of its final mass is N-linked carbohydrate (*1*). In most cases, mature clusterin is secreted from the cell; however, it remains intracellular in chickens (*2*). In humans, clusterin is found in blood plasma at about 100 *μ*g/mL and in seminal plasma at about 400 *μ*g/mL (*3*). Clusterin expression is increased in many experimental models of stress and disease states (*1*).

Many studies suggest that clusterin functions in vivo as a protective molecule. We recently reported that clusterin has an in vitro chaperone action that is similar to but more potent than that of the intracellular small heat shock proteins (*4*). Furthermore, we recently demonstrated that clusterin inhibits stress-induced precipitation of proteins in undiluted human serum, suggesting that the levels of clusterin in biological fluids such as plasma may affect the rate or extent of progression of diseases associated with abnormally high levels of protein precipitation (e.g., Alzheimer's, Creutzfeldt-Jakob, and Parkinson's diseases) (*5*). Other studies have shown that cells are protected from stresses by overexpression of clusterin, or by its addition to the medium surrounding cells (*1*). Recent studies with clusterin knock-out mice suggest that clusterin may protect cells from damage resulting from inflammatory reponses (*6*) but, in contrast, may enhance neurotoxicity in a model of hypoxic−ischemic injury (*7*). Collectively, these observations indicate that the interactions of clusterin with other biological molecules are likely to be of clinical importance.

The history of clusterin research has been notable for the rapid succession of reports describing new "specific" interactions of clusterin with a diverse array of native biological

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ligands. The strength of the evidence supporting the assertion that a particular interaction is specific varies from case to case, but the sheer number of putative binding ligands is impressive, including apoA-I, β-amyloid peptide, complement components, glutathione-*S*-transferase, LRP-2[1] (also known as gp330 or megalin, a member of the low-density lipoprotein receptor family), heparin, immunoglobulins, lipids, paraoxonase, prion peptide, SIC (streptococcal inhibitor of complement), the cell surface of *Staphylococcus aureus* isolates, and the TGF-β receptor (*1*). In addition to these interactions with native proteins, our recent work has shown that, during its chaperone action, clusterin binds to a very broad range of stressed, partly unfolded proteins (*5*).

It is unknown whether clusterin binds to all of these many ligands via one or more binding sites. Furthermore, the region(s) of clusterin involved in binding to any of the known ligands remain(s) to be identified, although peptides corresponding to residues 337−351 and 381−395 of clusterin have been shown to dose-dependently-inhibit clusterin-mediated aggregation of LLC−PK1 renal epithelial cells (*8*). As a first step toward determining how many types of ligand binding sites clusterin possesses, and those regions of the molecule that may be important in its binding interactions, we compared the chaperone and ligand binding activities of recombinant clusterin expressed in the yeast *Pichia pastoris* versus clusterin purified from human serum. These two forms of clusterin share many structural and functional features, but, in contrast to mature human serum clusterin, *Pichia*-expressed clusterin has variable proteolytic truncations of the C-terminal region of the α-chain and the N-terminal region of the β-chain. To further characterize the site(s) involved in clusterin binding interactions, we also tested whether the binding of clusterin to ligands could be inhibited by competitive binding with other clusterin ligands or by anti-clusterin monoclonal antibodies. Our results suggest that clusterin has independent binding sites for LRP-2, stressed proteins, and unstressed ligands.

MATERIALS AND METHODS

Materials. Human serum clusterin (*hs*Clus) was purified by immunoaffinity chromatography as previously described (*9*) and, when required, biotinylated by reaction with NHS-LC-biotin (Pierce, Rockford, IL), using standard methods. Glutathione-*S*-transferase (GST) from *Schistosoma japonicum* was prepared by thrombin cleavage of recombinant Jun leucine zipper−GST fusion protein and purified by GSH−agarose affinity chromatography as described by (*10*). Dithiothreitol (DTT), horseradish peroxidase (HRP), and streptavidin were purchased from Roche Molecular Biochemicals (Sydney, Australia). *N*-Glycosidase F was purchased from Boehringer Mannheim (IN). Phenylmethylsulfonyl fluoride (PMSF), *o*-phenylenediamine (OPD), and other chemicals and proteins were obtained from Sigma (St. Louis, MO). The cell line secreting the $IgG_1\kappa$ anti-clusterin monoclonal antibody G7 was a gift from Dr. B. Murphy (St. Vincent's Hospital, Melbourne, Australia). The 78E and 41D anti-clusterin monoclonal antibodies have been described before (*11*, *12*). The 78E antibody is α-chain-specific and reacts with a peptide corresponding to residues 1−23 of human clusterin (numbered from the N-terminus of the mature protein; J. Lakins, unpublished data). Dulbecco's modified Eagle's medium (DME) was purchased from Gibco BRL (Rockville, MD).

Expression of Human Clusterin in Pichia pastoris. (A) Cloning of Human Clusterin cDNA and Production of Pichia Transformants. The complete open reading frame of human clusterin cDNA, including the N-terminal secretory signal sequence, was amplified by polymerase chain reaction (PCR; using standard methods) from a template plasmid and subcloned into the *Pichia pastoris* expression vector pHIL-D2 (Invitrogen, San Diego, CA). For PCR, the forward primer was ACTAATTATTCGAAACGATGAAGACTCTGCTGCTGTTTG and consisted of 22 bases of the clusterin 5′ sequence beginning with the start codon (underlined) and a 5′ extension of 17 bases homologous to the 5′ untranslated region of the *P. pastoris* alcohol oxidase (AOX1) mRNA immediately upstream of the AOX1 start codon in pHIL-D2. The reverse primer was CAGTCATGTCTAAGGCGCATCTCACTCCTCCCGGTGCT and consisted of 21 bases of the clusterin 3′ sequence including the termination codon (underlined) and a 5′ extension of 17 bases homologous to the 5′ end of the AOX1 3′ transcription termination fragment in pHIL-D2. Subcloning of the PCR products into the pHIL-D2 vector was performed by homologous recombination in a *rec*A$^-$ *E. coli* host (*13*). The purified PCR products were transformed together with pHIL-D2 (linearized at the unique *Eco*RI site) into competent Top10F′ cells (Invitrogen). Low-frequency recombination between the homologous ends of the PCR product and the vector resulted in site-specific directional insertion. Insertion of the correct full-length clusterin sequence was confirmed by DNA sequencing using DyeDeoxy terminators on an ABI 370A automated sequencer. These constructs were then linearized at the unique *Sal*I site and used to transform *P. pastoris* spheroplasts as described by the manufacturer (Invitrogen). *Pichia* transformants were screened by immunodot assay for relative levels of clusterin expression (data not shown), and a high-expressing clone was selected for further work.

(B) Expression and Purification of Recombinant Human Clusterin. Culture and induction of recombinant protein expression in transformed *Pichia* clones were performed essentially as described by the manufacturer (Invitrogen). Briefly, a high-expressing *Pichia* transformant was inoculated into noninducing BMYG media, and grown in a shaking incubator for 1−2 days at 30 °C before pelleting the cells by centrifugation (50000*g* for 15 min) and resuspending them in BMMY media containing 0.5% (v/v) methanol (to induce recombinant protein expression) and 1% (w/v) heat-denatured casein (to minimize extracellular proteolysis). Induced cultures were incubated as above for 2 days, and recombinant *Pichia*-expressed clusterin (*rp*Clus) was purified from culture supernatant by immunoaffinity chromatography (*9*).

Enzymatic Deglycosylation of Clusterin and N-Terminal Sequencing. Preparations containing up to 50 μg of *rp*Clus or *hs*Clus were denatured by boiling for 10 min in 50 mM Tris-HCl, 1% (w/v) SDS, diluted with 150 mM Na_2HPO_4,

[1] Abbreviations: BSA, bovine serum albumin; BSA/PBS, 1% (w/v) bovine serum albumin in phosphate-buffered saline, pH 7.0; HDC, heat-denatured casein; HDC/PBS, 1% (w/v) heat-denatured casein in phosphate-buffered saline, pH 7.4; *hs*Clus, clusterin purified from human serum; LRP-2, lipoprotein receptor-related protein-2; PBS, phosphate-buffered saline; *rp*Clus, recombinant clusterin expressed in *Pichia pastoris*.

pH 8.3, to give a final concentration of 0.15% (w/v) SDS, and made 2 mM EDTA, 1 mM PMSF, 0.75% (v/v) NP40. These samples were then incubated overnight at 37 °C with 2.4 units of *N*-glycosidase F and diluted in sample buffer for SDS−polyacrylamide gel electrophoresis (SDS−PAGE; performed using standard methods), such that the final concentration of NP40 was less than 0.6% (v/v). In some experiments, recombinant clusterin was separated under reducing conditions by SDS−polyacrylamide gel electrophoresis and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). Automated Edman N-terminal sequence determinations were performed on selected PVDF-immobilized polypeptides with an Applied Biosystems Gas-Phase Sequencer (Models 470/120/900).

Protein Precipitation Assays. Stress-induced precipitation of substrate proteins was detected as turbidity, measured as an increase in absorbance at 360 nm (A^{360}) (*4*). The relative ability of *hs*Clus versus *rp*Clus to inhibit stress-induced protein precipitation was established by adding either type of clusterin to proteins undergoing stresses and measuring time-dependent changes in A^{360}. In all experiments, protein solutions were in 50 mM Na_2HPO_4, pH 7.0. In one type of experiment, protein precipitation was induced by heat. Solutions of ovotransferrin (1 mg/mL) or mixtures of ovotransferrin (1 mg/mL) with either *hs*Clus or *rp*Clus (at 75 μg/mL) were heated at 60 °C. The light scattering associated with ovotransferrin precipitation was measured every 3 min over 40 min using an automated seven-chambered diode-array spectrophotometer (Hewlett-Packard GMBH, Germany). In other experiments, protein precipitation was induced by reduction with DTT. Solutions of ovotransferrin (1 mg/mL) and lysozyme (0.25 mg/mL) were made 20 mM in DTT and then incubated for 3−6 h at 42 °C in the presence (or absence) of either *hs*Clus or *rp*Clus, both at 400 μg/mL. α-Lactalbumin (0.75 mg/mL) was treated similarly except that *hs*Clus or *rp*Clus was added to 200 μg/mL. No precipitation of ovotransferrin, lysozyme, or α-lactalbumin was measured in controls, in which DTT was omitted (data not shown). The light scattering associated with protein precipitation was measured at intervals with a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA).

Cell Culture. Murine F9 (embryonal carcinoma) cells were obtained from the American Type Culture Collection (Rockville, MD). Differentiated F9 cells were prepared essentially as described (*14*). Briefly, adherent cells were maintained in differentiation medium [10% (v/v) fetal bovine serum in DME containing 0.1 μM retinoic acid and 0.2 μM dibutyryl-cAMP] for 8 days. At this time, confluent cultures were subcultured into gelatin-coated 24 well plastic tissue culture dishes (Corning, NY) at about 1.8×10^5 cells/well. The cells were maintained in 500 μL of differentiation medium per well for 24−30 h prior to use in experiments measuring the binding of clusterin to the cell surface.

Clusterin Binding to the Surface of Differentiated F9 Cells. Purified *hs*Clus was labeled with ^{125}I using the chloramine T method (*15*). Labeled products were stored at 4 °C, and specific activity was corrected using the theoretical extent of decay at the time of use. The relative abilities of *rp*Clus and *hs*Clus to inhibit the binding of ^{125}I-labeled *hs*Clus to the surface of differentiated F9 cells were tested as follows. Differentiated F9 cells were washed once with serum-free medium consisting of 1% (v/v) NutridomaTM-SP (Boehringer-Mannheim, Indianapolis, IN) and 99% (v/v) binding buffer [DME containing 1.5% (w/v) BSA, pH 7.4] and then incubated for 2 h at 37 °C with 0.5 mL/well of the same medium. Plates were then placed on ice and media replaced with 240 μL/well of binding buffer containing 0.49 μg/mL ^{125}I-labeled *hs*Clus (specific activity 7.0×10^4 cpm/ng) and from 0- to 100=fold excess of unlabeled *hs*Clus or *rp*Clus. After incubation for 2.5 h at 4 °C, the cells were washed 4 times with ice-cold binding buffer and lysed with 300 μL/well of 0.1 M NaOH. Radioactivity of the lysates was measured in an LKB gamma counter. Nonspecific binding to F9 cells was considered to be counts bound in the presence of a 100-fold molar excess of unlabeled *hs*Clus and was subtracted from the total counts bound at each experimental condition. Specifically bound counts were normalized to the mean number of cells per well obtained from additional wells processed in parallel and converted to nanograms of specifically bound ^{125}I-labeled clusterin. The effect of the monoclonal antibody G7 on clusterin binding to differentiated F9 cells was tested by performing similar experiments to those described above in which ^{125}I-labeled *hs*Clus was preincubated with 52.5 μg/mL (a 47-fold molar excess) G7 antibody (or an isotype-matched control antibody) for 1 h on ice, before addition to F9 cells.

ELISA Assays. A series of experiments were performed to test the abilities of various molecules to inhibit clusterin binding to stressed and unstressed ligands.

(A) Clusterin Binding to Unstressed Ligands. Polystyrene 96 well microtiter plates (Corning, NY) were coated (50 μL/well, for 1−2 h at 37 °C or overnight at 4 °C) with human IgG (in 0.1 M Na_2CO_3, pH 9.5), Aβ_{1-40} (in 0.1 M Na_2CO_3, pH 8.6), or poly C9 in Tris-buffered saline (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 7.5). C9 was freshly polymerized prior to each experiment by incubating monomeric C9 at 100 μg/mL in TBS for 2 h at 37 °C containing 100 μM $ZnCl_2$ (*16*). After being coated, plates were blocked by a 1 h incubation at 37 °C with 100 μL/well of 1% (w/v) BSA in PBS, pH 7.0 (BSA/PBS). Where appropriate, bound biotinylated *hs*Clus was detected with 50 μL/well of a streptavidin/biotinylated HRP complex formed by mixing 1 μg/mL streptavidin and 1 μg/mL biotinylated HRP in BSA/PBS. The streptavidin/biotinylated HRP complex was incubated in the wells for 1.5 h at 37 °C, followed by three successive washes each with BSA/PBS, 0.1% (v/v) Triton X-100 in BSA/PBS, and finally BSA/PBS. In all experiments, plates were developed using OPD [2.5 mg/mL in 0.05 M citric acid, 0.1 M Na_2HPO_4, pH 5.0, containing 0.03% (v/v) H_2O_2] as substrate. Three different assays of this type were performed:

(1) Biotinylated *hs*Clus (0.5 μg/mL) was incubated in ELISA plate wells with 0−0.8 μg/mL *rp*Clus in PBS (pH 7.0). The wells had previously been coated as described above with Aβ_{1-40} (1 μg/mL) or IgG (1.5 μg/mL). (Data shown in Figure 4B.)

(2) *hs*Clus (1 μg/mL) was preincubated for 45 min at 37 °C with Zn^{2+}-polymerized C9 (0−0.4 μg/mL), and then added to wells coated with IgG (2.5 μg/mL; open squares), Aβ_{1-40} (2 μg/mL; open circles), or Zn^{2+}-polymerized C9 (1 μg/mL; solid circles). (Data shown in Figure 4C.)

(3) Biotinylated *hs*Clus (0.5 μg/mL) was preincubated for 45 min at 37 °C with 0−5 μM Aβ_{1-40} in PBS containing 10

mM MES (PBS/MES; pH 6.0). Samples were then diluted 1:1 with 2% (w/v) BSA in PBS/MES (pH 6.0) and added to wells coated as above with IgG (4 μg/mL) or poly C9 (5 μg/mL). (Data shown in Figure 4D.)

Bound clusterin was detected with G7 antibody followed by sheep anti-mouse Ig-HRP (SaMIgG-HRP; Silenus, Melbourne, Australia).

(B) Clusterin Binding to Stressed versus Unstressed GST. In one series of experiments, polystyrene 96 well microtiter plates (Sartorius, Adelaide, Australia) were coated with GST (20 μg/mL) in PBS (pH 7.4) for 1 h at either 60 °C or 37 °C. The plates were blocked with BSA/PBS as above. Mixtures of *hs*Clus (10 mg/mL) and GST (0−3 mg/mL) in PBS (pH 6.0) were preincubated for 1 h at 37 °C before adding to ELISA wells and incubating for 1 h at 37 °C. Bound clusterin was detected with G7 tissue culture supernatant, and bound primary antibodies were detected with SaM Ig-HRP and OPD substrate as described above.

(C) Clusterin Binding to Other Stressed Protein Ligands. Polystyrene 96 well microtiter plates (Sartorius, Adelaide, Australia) were coated with (i) 50 mM Na_2HPO_4, pH 7.0, containing 20 μg/mL ADH or cytochrome *c* (for 1 h at 60 °C) or BSA or lysozyme and 20 mM DTT (for 5 h at 37 °C), or (ii) 0.1 M $NaHCO_3$, pH 9.5, containing lysozyme (20 μg/mL) or insulin (100 μg/mL) for 1 h at 37 °C, followed by a 30 min incubation with 20 mM DTT in phosphate-buffered saline (PBS, pH 7.4). Plates were blocked by a 1 h incubation at 37 °C with 100 μL/well of PBS, pH 7.4, containing either 1% (w/v) heat-denatured casein (HDC/PBS) or 1% (w/v) BSA (BSA/PBS). Mixtures of clusterin (at 10 μg/mL) and GST (at 0−3 mg/mL), Aβ_{1-40} (at 0−2.0 μM), or heparin (at 0−50 μg/mL) in PBS/MES (pH 6.0) were preincubated for 1−2 h before adding to ELISA wells and incubated for 1 h at 37 °C. Bound clusterin was detected as described in (B), above.

(D) Effects of G7 Antibody on Clusterin Binding to Unstressed and Stressed Proteins. Biotinylated *hs*Clus (0.5 μg/mL) was preincubated for 30 min at room temperature with 0−50 μg/mL purified G7 antibody in PBS, pH 7.4, and then added to wells coated with (i) Aβ_{1-40} (4 μg/mL), IgG (5 μg/mL), or poly C9 (1.5 μg/mL), as above, (ii) G7 antibody (2 μg/mL in 0.1 M Na_2CO_3, pH 9.5) for 1−2 h at 37 °C, or (iii) ovotransferrin (at 20 μg/mL in 50 mM Na_2HPO_4, pH 7.0) for 1 h at 60 °C, or lysozyme (20 μg/mL), as above.

Data Analysis. The inhibitory effects of unlabeled clusterin on the binding of ^{125}I-labeled clusterin to F9 cells and the inhibitory effects of various ligands on the binding of clusterin to unstressed proteins in ELISA were analyzed using the equation:

$$B = B_{max}/(1 + K_i/[I]) \quad (1)$$

where B, B_{max}, K_i, and [I] represent, respectively, measured clusterin binding, clusterin binding in the absence of the inhibitor, the apparent inhibition constant, and the inhibitor concentration. This equation was fitted to the data by nonlinear regression analysis using Kaleidagraph v3.0.5 (Abelbeck Software, Reading, PA). Statistical comparisons between different apparent inhibition constants were made using Student's *t*-test; *t*-values with $p < 0.05$ were considered significant.

RESULTS

Molecular Analysis of rpClus. Human clusterin, complete with its N-terminal secretory signal sequence, was inducibly expressed in the yeast *Pichia pastoris*. Preliminary experiments indicated that *rp*Clus was (i) glycosylated, (ii) comprised of a mixture of disulfide-bonded heterodimers, and (iii) partially degraded by proteolysis (data not shown). The extent of (extracellular) proteolysis was reduced in subsequent experiments by adding 1% (w/v) HDC to the culture medium prior to induction. Additional strategies, such as shortening the induction period, did not further reduce the extent of proteolysis of *rp*Clus (data not shown). The yield of immunopurified *rp*Clus was estimated by competitive ELISA as 80−100 μg/ml of culture medium (*17*). Electrophoretic analyses of *rp*Clus expressed and purified on five separate occasions indicated that the quality of the preparations was very similar from batch to batch (data not shown). Immunopurified *rp*Clus was N-deglycosylated and then analyzed by two-dimensional (2-D) SDS−PAGE, in which proteins were separated under nonreducing conditions, followed by in-gel reduction and subsequent separation in the orthologous dimension (Figure 1). When analyzed by 2-D SDS−PAGE, *rp*Clus appears to be primarily a mixture of disulfide-linked dimers, each comprised of one α- and one β-chain (Figure 1B, region I). This interpretation was confirmed by (i) immunoblot analysis using the α-chain-specific monoclonal antibody 78E, and (ii) N-terminal sequence analysis of PVDF electroblots of selected spots (Figure 1, Table 1). Some lightly stained material was also detected lying on the diagonal (Figure 1B, region II); this probably represents minor clusterin species that have not been proteolytically cleaved. Last, some very weakly stained material was also detected migrating at low molecular mass (Figure 1B, region III); this represents low molecular mass clusterin peptides probably resulting from proteolytic degradation. The results are consistent with *Pichia* quantitatively cleaving *rp*Clus at one or more sites to generate disulfide-linked dimers that are subsequently secreted and partially "degraded" by extracellular proteases. The 26 kDa polypeptide detected by 2-D SDS−PAGE (spot 4, Figure 1B) had an N-terminal sequence matching that of mature *hs*Clus α-chain (i.e., with its signal peptide removed). Furthermore, the 28 kDa polypeptide (spots 1−3, Figure 1B) had an N-terminal sequence matching that of *hs*Clus β-chain. These polypeptides comigrated with N-deglycosylated hsClus α- and β-chains, respectively (data not shown), indicating that they are (at least approximately) full-length. Densitometric analysis of these and other 2-D gel spots shown in Figure 1 indicated that only about 13% of *rp*Clus was presumptively full-length.

The monoclonal antibody 78E (which binds to an N-terminal epitope in the α-chain) bound to all spots identified by N-terminal sequencing as α-chain (spots 5, 8, and 12, Figure 1B) and also to other minor spots not sequenced (spots 6, 7, and 14, Figure 1B; data not shown). Furthermore, the only N-terminal α-chain sequence detected matched the N-terminal sequence for mature *hs*Clus α-chain. Taken together, these observations suggest that smaller forms of the α-chain (spots 5, 7, 8, and 12, Figure 1B) resulted from C-terminal truncations. This interpretation is further supported by the observation that the collective yield of

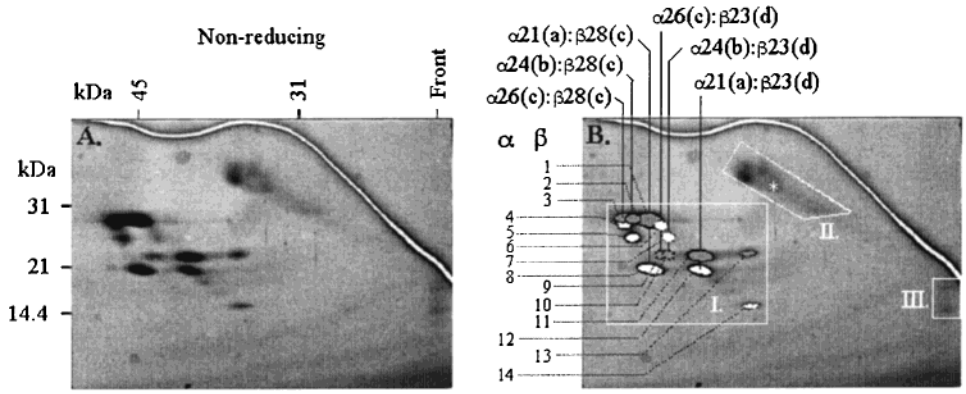

FIGURE 1: Coomassie blue-stained 2-D gel showing the separation of *rp*Clus following deglycosylation by treatment with *N*-glycosidase F. Protein was separated under nonreducing conditions in the first (horizontal) dimension, and then reduced in situ before being separated in the second (vertical) dimension. (A) Image of stained gel. A crack is visible running from the top left corner to the middle of the right side of the gel; this appeared during gel drying. (B) Image of the same gel annotated to show the identity of various protein species. The majority of *rp*Clus lies off the diagonal in a pattern of (vertically) collinear pairs of spots (I), consistent with it being a mixture of disulfide-linked dimers. Some lightly stained material was detected lying on the diagonal (II), and some very weakly stained material was also detected migrating at low molecular mass (III). The position of *N*-glycosidase F is indicated by "*". Preliminary identification of α- and *β*-chains was performed by immunoblotting using the anti-clusterin monoclonal antibody 78E (which is α-chain-specific)—white-filled spots (putative α-chain) were reactive with 78E; gray-filled spots (putative *β*-chain) did not react with 78E. To further characterize the molecular species, selected regions of PVDF electroblots containing one or more spots (individual spots labeled 1−14) were subjected to N-terminal sequencing (Table 1). This analysis confirmed that the dimers each consist of one α- and one *β*-chain. Paired subunits are shown connected by a vertical line to a descriptor of the type α*XX*(a):*βYY*(b), where α and *β* designate the chain, *XX* and *YY* represent the respective masses in kDa, and (a) and (b) represent the presumptive cleavage sites that generate these species (shown in Figure 2). Minor spots, shown in dotted outline (6, 7, 9, 10, 13, and 14), were identified by immunoblotting only.

Table 1: N-Terminal Sequencing Data for Protein Spots Shown in Figure 1

spots	α-chain	*β*-chain
1	D_1QTV[a]...(0.42 ± 0.21 pmol/cycle)[b]	S_1LMP...(3.92[a] ± 1.19 pmol/cycle)[b]
2 and 3	D_1QTV...(0.96 ± 0.23 pmol/cycle)	S_1LMP...(2.96 ± 0.58 pmol/cycle)
4 and 5	D_1QTV...(1.32 ± 0.85 pmol/cycle)	
8	D_1QTV...(3.72 ± 2.41 pmol/cycle)	
11		S_{37}PAF... (3.13 ± 1.00 pmol/cycle)
12	D_1QTV...(2.11 ± 0.92 pmol/cycle)	

[a] First four amino acids of the identifiable N-terminus, the chain, and the position in the chain (subscript) relative to the corresponding mature N-terminus of *hs*Clus. [b] Average yield and standard deviation through 10 sequencing cycles of the corresponding N-terminus.

polypeptides with an N-terminus identical to mature *hs*Clus α-chain was roughly equivalent to the sum of yields for the two forms of *β*-chain (see below). Two N-terminal *β*-chain sequences were detected—one matched the N-terminus of *hs*Clus *β*-chain; the other corresponded to an N-terminal 36 amino acid truncation of *hs*Clus *β*-chain. The latter N-terminus was detected only for the 23 kDa polypeptide (spots 9−11 and 13, Figure 1B). This N-terminal truncation easily accounts for the approximately 5 kDa decrease in molecular mass, relative to the 28 kDa full-length *β*-chain (i.e., spots 1−3, Figure 1B). Densitometric analysis indicated that 87% of *rp*Clus was comprised of species with proteolytic truncations of either or both the α- and *β*-chains (about 41% was comprised of species with truncations of both the α- and *β*-chains) (data not shown).

Identification of Major Proteolytic Cleavage Sites in rpClus. The above molecular analyses localized the major proteolytic cleavage sites in *rp*Clus to the Arg^{205}−Ser^{206} and His^{240}−Ser^{241} bonds (numbered from the N-terminal residue of the mature protein; indicated by arrows labeled "c" and "d", respectively, in Figure 2), and two other sites at the C-terminus of the α-chain (arrows labeled "a" and "b", Figure 2). Highly purified and homogeneous preparations are desirable to investigate the structural basis of protein function. However, the proteolytic truncations of specific regions of *rp*Clus gave us the opportunity to test whether those regions of clusterin are important in its known interactions with other molecules. Thus, if the C-terminus of the α-chain and/or the N-terminus of the *β*-chain are important in clusterin's chaperone action or specific binding interactions, then it would be expected that, compared to *hs*Clus, *rp*Clus would show reduced activity.

rpClus Has Similar in Vitro Chaperone Activity to hsClus. We tested the relative abilities of *hs*Clus and *rp*Clus to inhibit the heat-induced precipitation of ovotransferrin, and the reduction-induced precipitation of ovotransferrin, α-lactalbumin, or lysozyme. In all cases tested, the inhibition of stress-induced protein precipitation effected by *hs*Clus and *rp*Clus at equivalent concentrations was virtually identical (Figure 3A−D). In each case, the *hs*Clus:stressed protein ratio tested was sufficient to inhibit between 55 and 80% of the protein precipitation that occurred in the absence of clusterin. Higher concentrations of clusterin caused greater inhibition of stress-induced protein precipitation (data not shown). In the experiment measuring DTT-induced precipitation of ovotransferrin, the small decrease in absorbance measured commencing at about 130 min corresponded to a visible settling of aggregates from solution (Figure 3B).

Binding of hsClus and rpClus to Unstressed Proteins. Both *hs*Clus and *rp*Clus gave dose-dependent inhibition of the binding of ^{125}I-labeled *hs*Clus to the LRP-2 receptor expressed on the surfaces of differentiated F9 cells (Figure 4A).

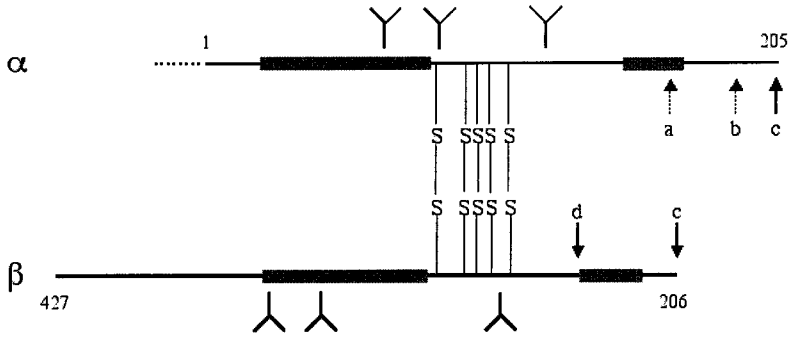

FIGURE 2: Major sites of *rp*Clus proteolysis. The arrows 'a' to 'd' show the major sites of proteolysis of *rp*Clus derived from analyses of data shown in Figure 1, superimposed on a linear (α- and *β*-chain) antiparallel model of clusterin structure inferred from the pattern of interchain disulfide bonds (*22*). Numbers of amino acid residues (starting from the N-terminus of the mature protein) are indicated; the signal peptide is indicated as a dotted line. Shaded boxes identify predicted amphipathic α-helices (*23, 24*), and "Y"s identify sites of N-linked glycosylation (*25*). Sites of proteolysis identified directly from N-terminal sequence data are indicated by solid arrows, while the approximate positions of those identified based on molecular masses of α-chains (containing the normal mature N-terminus) determined by SDS−PAGE are indicated by dotted arrows. Arrow 'c' is the single "normal" site of proteolytic cleavage (at the Arg^{205}−Ser^{206} bond) that occurs prior to secretion of clusterin from mammalian cells.

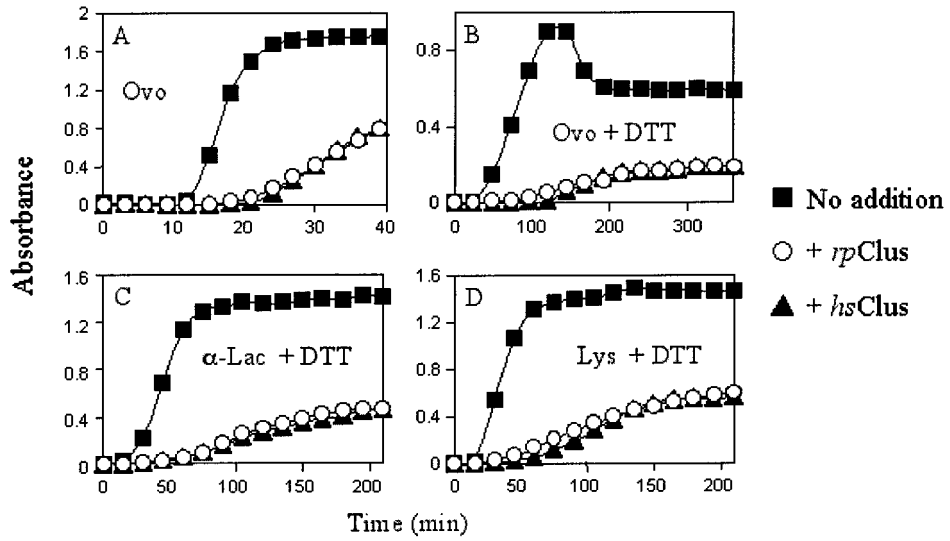

FIGURE 3: Inhibition of stress-induced protein precipitation by different forms of clusterin. Ovotransferrin (Ovo) was stressed by heating at 60 °C (A) or 42 °C in the presence of 20 mM DTT (B). Both α-lactalbumin (α-Lac; C) and lysozyme (Lys; D) were stressed as in (B). Protein precipitation was measured as turbidity (absorbance at 360 nm) as a function of time, with no additions (■), or with equivalent additions of *hs*Clus (▲) or *rp*Clus (○) as described under Materials and Methods. The data shown are means ± standard deviations of triplicates. In most cases, the error bars are smaller than the data symbols.

When eq 1 was fitted to these data by nonlinear regression, there was no significant difference between the apparent inhibition constants (K_i) for *rp*Clus and *hs*Clus (Table 2), implying that these two forms of clusterin bind to LRP-2 with experimentally indistinguishable affinities. In contrast, *rp*Clus was significantly less effective than *hs*Clus at inhibiting the binding of biotinylated *hs*Clus to both $A\beta_{1-40}$ and IgG in ELISA (Figure 4B). Nonlinear regression analysis showed that, in inhibiting the binding of biotinylated *hs*Clus to $A\beta_{1-40}$ and IgG, the apparent K_i values for *rp*Clus were 4.5- and 4.2-fold higher, respectively, than those for *hs*Clus (Table 2), implying that *rp*Clus binds to both $A\beta_{1-40}$ and IgG with significantly lower affinity than does *hs*Clus.

To investigate whether clusterin has one or more binding sites for unstressed ligands, we tested the extent of competitive inhibition of clusterin binding to unstressed ligands by other unstressed ligands. Preincubation with poly C9 inhibited, in a dose-dependent manner, the subsequent binding of biotinylated *hs*Clus to poly C9, IgG, and $A\beta_{1-40}$ (Figure 4C). Nonlinear regression analysis showed that in the latter two cases, the apparent K_i values were not significantly different from each other but that the apparent K_i for inhibition of binding to poly C9 was slightly (about 25%) lower than that for binding to IgG (Table 2). Furthermore, preincubation with $A\beta_{1-40}$ also inhibited binding of biotinylated *hs*Clus to the same three unstressed ligands, with statistically indistinguishable inhibition constants (Figure 4D, Table 2).

Interactions with Unstressed Ligands Have Little Effect on Clusterin Binding to Stressed Proteins. Clusterin binds relatively weakly to unstressed GST but binds more strongly when GST is heat-stressed (*4*). Preincubation with unstressed GST produced dose-dependent inhibition of the subsequent binding of *hs*Clus to unstressed GST but had no effect on its binding to heat-stressed GST (Figure 5A). The observation that GST in solution only partially inhibited the binding of clusterin to unstressed GST on the ELISA tray may reflect the propensity of GST to polymerize (*18*). When incorporated into solution-phase clusterin−GST complexes, clusterin would be unable to bind to adsorbed GST because its GST

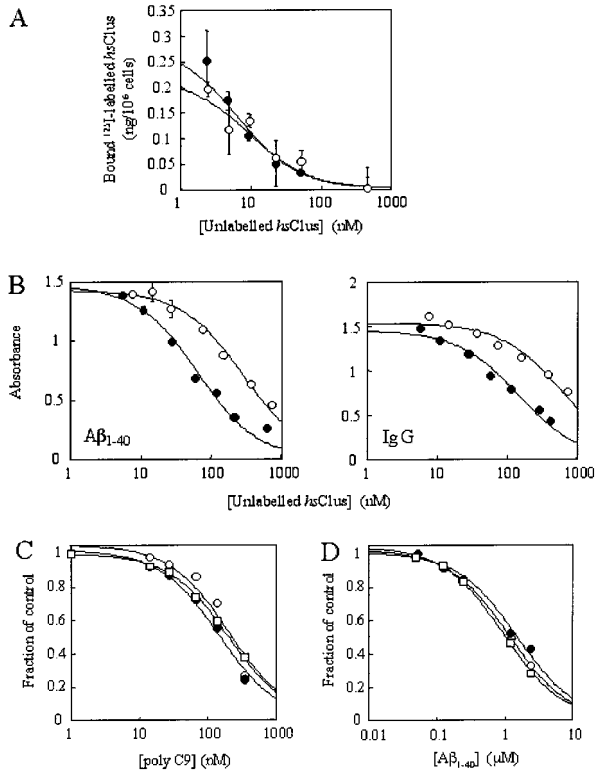

FIGURE 4: (A) Inhibition of the binding of ^{125}I-labeled *hs*Clus to LRP-2 expressed on the surface of differentiated F9 cells. The binding of ^{125}I-labeled *hs*Clus to LRP-2 on F9 cells in the presence of the indicated concentrations of unlabeled *hs*Clus (solid symbols) or *rp*Clus (empty symbols) was measured as described under Materials and Methods. The data shown are means ± standard deviations of triplicates and are representative of several independent experiments. The curves shown were obtained by regression of eq 1 onto the data. (B) Inhibition of the binding of biotinylated *hs*Clus to Aβ_{1-40} and IgG in ELISA. The binding of biotinylated *hs*Clus to Aβ_{1-40} (left panel) and to IgG (right panel) in the presence of the indicated concentrations of unlabeled *hs*Clus (solid symbols) or *rp*Clus (empty symbols) was measured by ELISA as described under Materials and Methods. The data shown are means ± standard deviations of triplicates and are representative of two independent experiments. The curves shown were obtained by regression of eq 1 onto the data. (C and D) Inhibition of the binding of biotinylated *hs*Clus to Aβ_{1-40}, poly C9, and IgG in ELISA. The binding of biotinylated *hs*Clus to poly C9 (●), Aβ_{1-40} (○), or IgG (□) in the presence of the indicated concentrations of poly C9 (C) or Aβ_{1-40} (D) was measured by ELISA as described under Materials and Methods. The data shown are single measurements and are representative of two independent experiments. The curves shown were obtained by regression of eq 1 onto the data.

binding site would already be occupied. However, clusterin−GST complexes in solution may still bind to adsorbed GST via a GST−GST interaction. In this event, solution-phase GST could produce only partial inhibition of the total amount of clusterin binding to absorbed GST, as seen in Figure 5A.

In similar experiments, preincubation with unstressed GST had no effect on the subsequent binding of *hs*Clus to heat-stressed ADH or cytochrome *c*, or lysozyme stressed by reduction, but did cause partial inhibition of its binding to BSA stressed by reduction (Figure 5B). However, in the latter case, binding was inhibited by less than 30% and, for concentrations of GST between 1 and 3 mg/mL, did not show a dose-dependence. The reason(s) for this limited inhibition is (are) unclear, but it might arise because of steric factors. The binding of unstressed GST to one or more clusterin binding sites (which is probably saturated at a GST concentration of less than 1 mg/mL) might partially sterically hinder the binding of stressed BSA (the largest of the stressed protein ligands tested) to other clusterin binding site(s). We also tested two more unstressed clusterin ligands for their ability to competitively inhibit the binding of clusterin to stressed proteins. Preincubation with Aβ_{1-40} or heparin had no effect on the subsequent binding of *hs*Clus to, respectively, lysozyme or insulin stressed by reduction (Figure 6). Preincubation with Aβ_{1-40} inhibited the subsequent binding of *hs*Clus to Aβ_{1-40} adsorbed to ELISA plates (Figure 4D), confirming that clusterin−Aβ_{1-40} interactions occurred during the preincubation step. As heparin does not bind effectively to ELISA plates, native gel electrophoresis was used to confirm that clusterin complexed with heparin during the preincubation step (data not shown).

Monoclonal Antibody G7 Inhibits Binding of Clusterin to LRP-2 but Not to Other Ligands. Preincubating ^{125}I-labeled *hs*Clus with G7 antibody or an excess of unlabeled *hs*Clus potently inhibited its binding to LRP-2 expressed on the surface of differentiated F9 cells (Figure 7A). An isotype control antibody had no effects under the same conditions (data not shown). In contrast, at concentrations up to in excess of 50 μg/mL, G7 antibody had little effect on the binding of biotinylated *hs*Clus to Aβ_{1-40}, IgG, or poly C9 in ELISA (Figure 7B−D). The very slight increase in absorbance detected in wells containing G7 (most noticeable in Figure 7B,D) may have resulted from nonspecific binding of clusterin−G7 complexes to the wells. At the highest concentration of G7 tested, compared with wells containing no G7, there was about a 10% decrease in clusterin binding to poly C9 (Figure 7D). The reason(s) for this effect is (are) uncertain, but it is clearly a minor effect. Under the same conditions, preincubation with G7 inhibited the binding of biotinylated *hs*Clus to solid-phase adsorbed G7 (Figure 7B−D), confirming that G7 was binding to its specific *hs*Clus epitope during the solution-phase preincubation. Last, in similar experiments, preincubation with G7 had no effect on the binding of *hs*Clus to stressed ovotransferrin or lysozyme (Figure 7E,F).

DISCUSSION

Electrophoretic analyses and N-terminal sequencing of purified *rp*Clus indicated that, like *hs*Clus, the purified product consists of an N-glycosylated, disulfide-linked heterodimer, cleaved at the Arg^{205}−Ser^{206} bond. However, unlike *hs*Clus, purified *rp*Clus contained a mixture of species with a series of truncations of the C-terminus of the α-chain and the N-terminus of the β-chain (Figure 2). We exploited these differences between *hs*Clus and *rp*Clus to determine whether those regions truncated in *rp*Clus were important in the chaperone action of clusterin or its binding to a variety of protein ligands. It was shown that *hs*Clus and *rp*Clus have virtually identical chaperone activity, as judged by their abilities to inhibit stress-induced protein precipitation in vitro (Figure 3). In addition, compared with *hs*Clus, *rp*Clus was shown to bind similarly to LRP-2 expressed at the surface of differentiated F9 cells but with substantially lower affinity to Aβ_{1-40} and IgG (Table 2). One implication of these results is that the precise composition of the sugar chains attached to clusterin, which would be different for *hs*Clus and *rp*Clus, is not critical for the chaperone action or for binding to LRP-

Table 2: Results of Nonlinear Regression Analysis[a]

data	target	ligand	inhibitor	K_i (nM)	significance of differences
Figure 4A	LRP-2	^{125}I-labeled *hs*Clus	*hs*Clus	6.5 ± 1.1	N/A
Figure 4A	LRP-2	^{125}I-labeled *hs*Clus	*rp*Clus	9.4 ± 1.5	not significant compared to *hs*Clus binding to LRP-2
Figure 4B	Aβ_{1-40}	biotinylated *hs*Clus	*hs*Clus	64 ± 11	N/A
Figure 4B	Aβ_{1-40}	biotinylated *hs*Clus	*rp*Clus	286 ± 33	significant compared to inhibition by *hs*Clus ($p < 0.001$)
Figure 4B	IgG	biotinylated *hs*Clus	*hs*Clus	144 ± 22	N/A
Figure 4B	IgG	biotinylated *hs*Clus	*rp*Clus	605 ± 102	significant compared to inhibition by *hs*Clus ($p < 0.005$)
Figure 4C	poly C9	biotinylated *hs*Clus	poly C9	147 ± 17	N/A
Figure 4C	IgG	biotinylated *hs*Clus	poly C9	204 ± 6.5	marginally significant compared to inhibition of binding to poly C9 ($p < 0.025$)
Figure 4C	Aβ_{1-40}	biotinylated *hs*Clus	poly C9	208 ± 55	not significant compared to inhibition of binding to poly C9
Figure 4D	Aβ_{1-40}	biotinylated *hs*Clus	Aβ_{1-40}	1491 ± 204	N/A
Figure 4D	IgG	biotinylated *hs*Clus	Aβ_{1-40}	1276 ± 30	not significant compared to inhibition of binding to Aβ_{1-40}
Figure 4D	poly C9	biotinylated *hs*Clus	Aβ_{1-40}	933 ± 45	not significant compared to inhibition of binding to Aβ_{1-40}

[a] Equation 1 was fitted to the indicated data sets as described, and the significance of differences between K_i values was assessed as described under Materials and Methods. N/A: Not applicable.

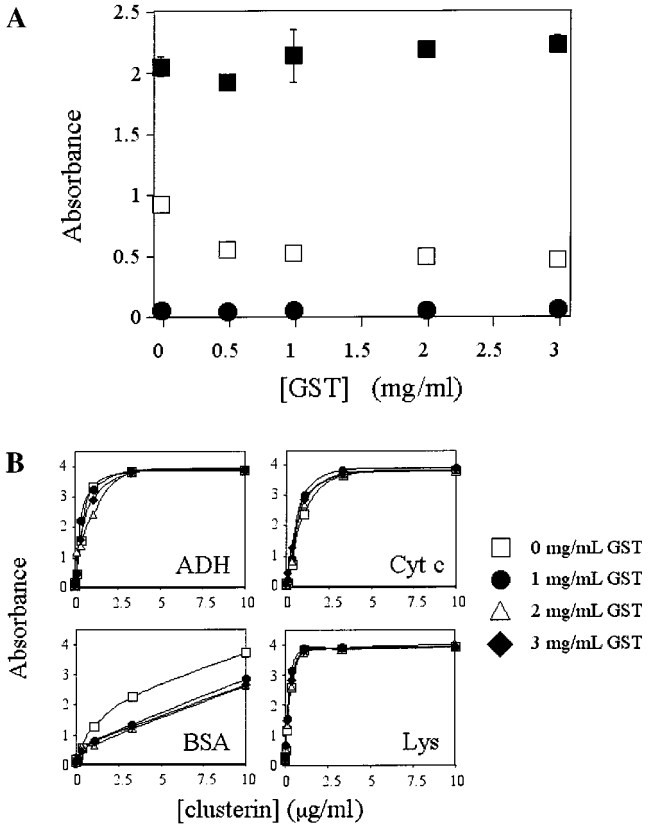

FIGURE 5: Effects of preincubation with GST on the subsequent binding of *hs*Clus to target proteins in ELISA. *hs*Clus was incubated with GST at the indicated concentrations, and its binding to the following ligands was measured in ELISA as described under Materials and Methods: (A) unstressed GST (□), heat-stressed GST (■), or unstressed BSA (●); (B) heat-stressed ADH or cyt *c*, or BSA or lysozyme (Lys) stressed by reduction. In (B), binding of *hs*Clus to unstressed ADH, cyt *c*, BSA, or lysozyme was negligible (data not shown). Following coating of reduced proteins, incubation of the wells with 10 mM iodoacetamide (to block free sulfhydryl groups) had no effect on the subsequent binding of *hs*Clus [(*4*); and data not shown]. The data shown are means ± standard deviations of triplicates and are representative of several independent experiments. In some cases, the error bars are smaller than the data symbols.

2. However, we speculate that the overall hydrophilicity of the sugar chains may help clusterin solubilize stressed protein substrates.

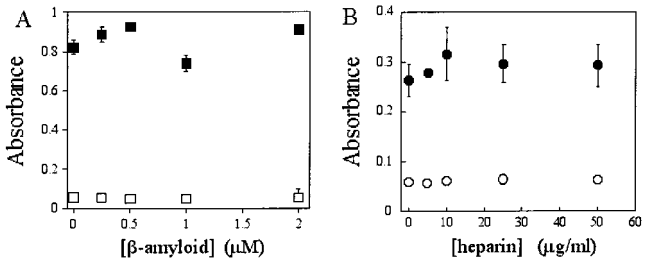

FIGURE 6: Effects of preincubation with Aβ_{1-40} and heparin on the subsequent binding of *hs*Clus to target proteins in ELISA. *hs*Clus was incubated with Aβ_{1-40} (A) or heparin (B) at the indicated concentrations, and its binding to the following ligands was measured in ELISA as described under Materials and Methods: (A) unstressed lysozyme (□) or lysozyme stressed by reduction (■); (B) unstressed insulin (○) or insulin stressed by reduction (●). The data shown are means ± standard deviations of triplicates and are representative of several independent experiments. In many cases, the error bars are smaller than the data symbols.

Collectively, the results suggest the following in the clusterin molecule: (i) Either the C-terminus of the α-chain and/or the N-terminus of the β-chain contains binding site-(s) for Aβ_{1-40} and IgG, or that proteolytic truncation of these regions affects other parts of the molecule that contain such binding sites. (ii) The region(s) responsible for the chaperone action and for binding to LRP-2 is (are) different from those involved in binding to Aβ_{1-40} and IgG and are unlikely to be located in the C-terminus of the α-chain or the N-terminus of the β-chain. When considering these interpretations, it is important to note that we cannot exclude the possibility that the difference in glycosylation between *hs*Clus and *rp*Clus might affect their respective abilities to bind to Aβ_{1-40} and IgG, even though this difference has no effect on their (equivalent) binding to stressed proteins and LRP-2. Furthermore, clusterin is known to reversibly aggregate in solution (*19*). It is conceivable that the identified proteolytic truncations of *rp*Clus might affect its level of aggregation, thereby reducing its ability to bind to Aβ_{1-40} and IgG. However, this is unlikely for two reasons. First, when *rp*Clus and *hs*Clus were analyzed by native gradient gel electrophoresis, the most abundant protein species in each case migrated with similar mobility (data not shown). This suggests that there was no gross difference in the extent of aggregation between the two forms of clusterin. Second,

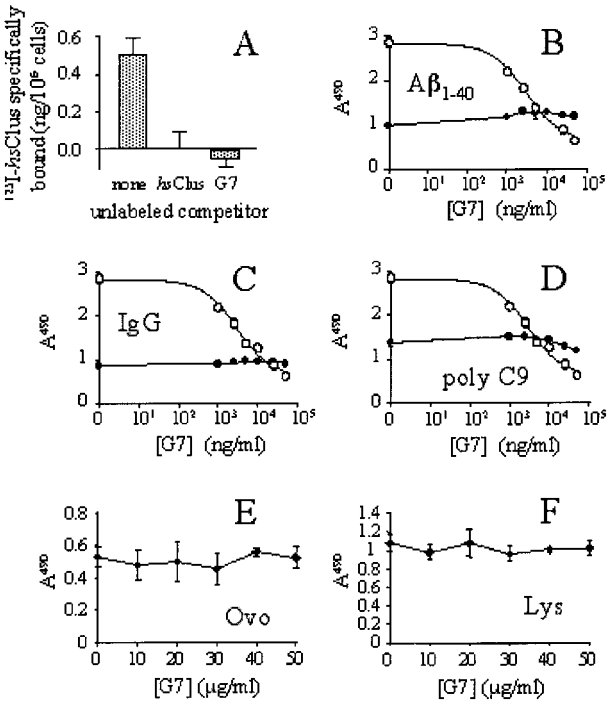

FIGURE 7: Effects of preincubation with monoclonal antibody G7 on the subsequent binding of *hs*Clus to F9 cells and target proteins. (A) Histogram plot showing the effects of unlabeled *hs*Clus and G7 monoclonal antibody on the binding of ^{125}I-labeled *hs*Clus to LRP-2 expressed on the surface of differentiated F9 cells. (B−F) Plots showing results from ELISA measuring the effects of preincubation with unlabeled *hs*Clus (○; B−D) or G7 monoclonal antibody (●; B−F) on the subsequent binding of biotinylated *hs*Clus to Aβ_{1-40} (B), IgG (C), poly C9 (D), heat-stressed ovotransferrin (E), or lysozyme stressed by reduction (F). In each case, the data shown are means ± standard deviations of triplicates. In many cases, the error bars are smaller than the data symbols.

Matsubara et al. (1995) have shown that both monomeric (85 kDa) and dimeric (170 kDa) forms of clusterin found in purified preparations of human serum clusterin bind with high affinity to Aβ_{1-40} to form stable 1:1 complexes (*20*). Thus, at least in the case of Aβ_{1-40}, the level of clusterin oligomerization does not appear to substantially affect its binding to ligand.

To further characterize site(s) involved in clusterin binding interactions, we tested for reciprocal competitive inhibition interactions between individual clusterin ligands. Solution-phase poly C9 and Aβ_{1-40} both competitively inhibited *hs*Clus binding to solid-phase adsorbed poly C9, Aβ_{1-40}, and IgG, with apparent inhibition constants for poly C9 and Aβ_{1-40} in the ranges 150−200 nM and 1−1.5 μM, respectively (Figure 4C,D, Table 2). The finding that poly C9 and Aβ_{1-40} inhibited the binding of *hs*Clus to each of poly C9, Aβ_{1-40}, and IgG with similar potency suggests that all of these ligands bind to the same clusterin binding site(s), or to binding sites that are physically close in three-dimensional space. We next tested whether interactions of clusterin with unstressed ligands inhibited its subsequent binding to stressed proteins in ELISA. *hs*Clus was shown to interact in solution with GST, Aβ_{1-40}, and heparin (Figures 4D and 5A, and data not shown) but these interactions had little or no effect on its binding to a variety of stressed proteins tested (Figures 5 and 6). These results are consistent with the suggestion that arose from comparisons of the chaperone and binding activities of *hs*Clus and *rp*Clus (see above) that regions of the clusterin molecule important in its chaperone action, including those regions that bind to stressed proteins, are different than those that bind to unstressed ligands.

As an additional approach to characterize site(s) involved in clusterin binding interactions, we tested the ability of monoclonal anti-clusterin antibodies to inhibit the binding of *hs*Clus to LRP-2 expressed on the surface of F9 cells, or to a number of unstressed or stressed proteins. The G7 antibody potently inhibited binding of *hs*Clus to LRP-2 (Figure 7A) but had little effect on its binding to any of the unstressed or stressed proteins tested (Figure 7B−F), indicating that the clusterin binding site for LRP-2 is distinct from those for the other ligands tested. The precise position and sequence of the G7 epitope are unknown. However, it is known that the G7 epitope is very sensitive to reduction (*1*), suggesting that it may lie in close proximity to the highly conserved, disulfide-bonded region of clusterin. Furthermore, immunoblotting studies have demonstrated that, following reduction, G7 shows weak reactivity with the α-chain but not with the β-chain (data not shown). Thus, it is likely that the G7 epitope is positioned (at least partly) within or adjacent to the disulfide-bonded region of the α-chain. This epitope may overlap the binding site(s) for LRP-2, or G7 antibody may sterically inhibit their binding to clusterin. Allowing for either interpretation, these results suggest that the binding site(s) for LRP-2 is (are) probably in or adjacent to the disulfide-bonded region of the α-chain, or is (are) adjacent to this region in three-dimensional space.

To summarize, taken together, our results suggest that clusterin has at least three distinct types of binding sites:

(1) One or more sites that bind to unstressed ligands such as Aβ_{1-40}, poly C9, and IgG, which may be located in the C-terminus of the α-chain and/or the N-terminus of the β-chain (possibly involving the amphipathic α-helices in these regions; Figure 2).

(2) One or more sites that bind to stressed proteins and are important in the chaperone action. These sites are unlikely to occur in the C-terminus of the α-chain or the N-terminus of the β-chain.

(3) One or more sites that bind to the cell surface receptor LRP-2. These sites may be located in or adjacent to (in three-dimensional space) the highly conserved, disulfide-bonded region of the α-chain.

We currently have insufficient data to reliably assign exact positions to any of these binding sites. To more precisely identify regions of the clusterin molecule important in its various binding interactions, we have commenced structure/function studies of mutant recombinant clusterin molecules.

Accepting that clusterin has at least three distinct types of binding sites, what are the physiological functions of these sites? Clearly, binding to stressed proteins is important in the chaperone action of clusterin and is expected to be critical in its ability to bind to and solubilize proteins that have partly unfolded as a result of stress. As an additional, possibly complementary function, it has been suggested that clusterin binds to toxic hydrophobic molecules in the extracellular environment and carries these to cells expressing LRP-2 for uptake and degradation. It has been demonstrated that, following binding to cells expressing LRP-2, clusterin and clusterin−Aβ_{1-40} complexes are internalized by receptor-mediated endocytosis and degraded by lysosomal enzymes (*14, 21*). The uptake of clusterin−Aβ_{1-40} complexes and the

putative role for clusterin as a scavenger and disposal vehicle are consistent with our demonstration that the binding site(s) for LRP-2 is (are) distinct from those for other ligands. Thus, clusterin may bind to exposed regions of hydrophobicity on a variety of potentially harmful (stressed or unstressed) ligands and subsequently bind by a separate site to the LRP-2 receptor on the surface of cells that mediate safe disposal of the cargo. However, confirmation that clusterin acts as a "molecular scavenger" in vivo will require further investigations.

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