Characterization of the Heparin-Binding Properties of Human Clusterin

Greg J. Pankhurst, Christine A. Bennett, and Simon B. Easterbrook-Smith*

Department of Biochemistry University of Sydney, Sydney NSW 2006, Australia

Received September 24, 1997; Revised Manuscript Received January 14, 1998

ABSTRACT: Clusterin is a highly conserved mammalian glycoprotein which has been predicted to contain heparin-binding sites. We tested this prediction by studying the interactions between heparin and clusterin using ELISA and heparin affinity chromatography methodologies. Two forms of biotinylated heparin were used in ELISA: heparin which had been directly biotinylated with a biotin-*N*-hydroxysuccinimide ester and heparin which had been activated using epichlorohydrin and 1,6-diaminohexane prior to biotinylation. Both gave dose-dependent increases in ELISA signal with increasing concentrations of biotinylated heparin, with the latter giving signals an order of magnitude greater than the former. There was a dose-dependent increase in the ELISA signal from bound biotinylated heparin with increasing concentrations of plate-bound clusterin. The apparent affinity constant for binding of biotinylated heparin to plate-bound clusterin at pH 6.0 was estimated as 0.06 ± 0.02 μM. Unlabeled heparin blocked the binding of biotinylated heparin to clusterin over a concentration range similar to that of the binding of biotinylated heparin to plate-bound clusterin. The binding of biotinylated heparin to clusterin was independent of the presence or absence of Ca^{2+}. The binding of biotinylated heparin to plate-bound clusterin increased with decreasing pH over the range 5.5−8.0 and was characterized by an apparent pK_a of 6.9. Clusterin in human serum bound to heparin−Sepharose at pH 6.0 but not at pH 7.4. Dot-blot experiments showed that one of the polypeptide chains of clusterin which had been reduced and alkylated under denaturing conditions bound to heparin−Sepharose. This chain was identified as the α chain from its N-terminal amino acid sequence.

Clusterin (also known as apolipoprotein J, TRPM-2, SGP-2, gpIII, and gp80) was first described in 1983 as a secreted glycoprotein produced by ram Sertoli cells and which was able to elicit clustering of various cell types (*1*). The mature form of secreted clusterin is a disulfide-linked heterodimer of α and β polypeptide chains, each ≈ 40 kDa, produced by cleavage of the primary translation product at its arg205−ser206 bond (*2*). This form of clusterin is present in most body fluids including serum, semen, saliva, urine, milk, and cerebrospinal fluid (*1*, *3*, *4*). Recently, an intracellular form of clusterin, apparently produced by differential translation, has been identified. This form of clusterin interacts with the cytoplasmic domains of TGF-β[1] receptors and localizes to the nuclei of epithelial cells following treatment with TGF-β (*5*, *6*).

The physiological functions of clusterin remain enigmatic. It has been implicated in a diverse array of physiological processes, including sperm maturation, regulation of complement activation, lipid transport, endocrine secretion, tissue remodeling, and programmed cell death. Currently there is no consensus as to which of these represent genuine physiological functions (reviewed in ref *7*). The range of biological molecules which have been reported to bind to clusterin is similarly diverse. These include complement proteins C7, C8, and C9 (*8*, *9*), apolipoprotein A−I (*10*), immunoglobulins (*11*, *12*), Alzheimer's β-amyloid peptide (*13*), and Streptococcal protein SIC (*14*).

Analyses of the amino acid sequence of clusterin have shown that clusterin homologues in different mammals have substantial sequence similarity. For example, there is 75% sequence identity between the human and murine forms of the protein (*15*, *16*). Sequence analyses have also led to predictions of a number of structural and functional properties of clusterin. These include predictions that clusterin is N-glycosylated, that it is a nucleotide-binding protein, that it contains amphipathic α-helices, and that it is a heparin-binding protein (*2*, *17*, *18*). Only the first two of these predictions have been tested experimentally; studies of the expression of clusterin by HepG2 cells have shown that it is N-glycosylated (*19*) and the observation that clusterin does not bind to AMP−Sepharose implies that it is not a nucleotide-binding protein (*20*).

The heparin-binding motifs proposed by Cardin and Weintraub (*21*), XBBXBX and XBBBXXBX, where B is either arg or lys and X is any amino acid, were used to predict four potential heparin-binding domains in clusterin (*2*). Two of these are in the α chain (residues 45−60 and 190−196), and two are in the β chain (residues 402−408 and 420−427). More recent analyses have shown that some heparin-binding proteins do not contain the Cardin and Weintraub motifs in their heparin-binding sites (*22*, and references therein). Using molecular graphics techniques the spatial distribution of basic amino acid residues were compared

* Author to whom correspondence should be addressed. Telephone: (+) 612 9351 3905. Fax: (+) 612 9351 4726. E-mail: sbe@biochem.usyd.edu.au.

[1] Abbreviations: TGF-β, transforming growth factor β; b-heparin, biotinylated heparin; MAb, monoclonal antibody; NHS, *N*-hydroxysuccinimide.

S0006-2960(97)02367-2 CCC: $15.00
Published on Web 03/20/1998

within peptides with known heparin-binding activity. This led to the proposal that two basic residues about 20 Å apart may define the heparin-binding motif of α-helical peptides (*22*). The predicted heparin-binding site between residues 45 and 60 of the α chain of clusterin satisfies this motif, because the PredictProtein neural network (*23*) predicts that this region of clusterin forms an α-helix within which arg46 and lys59 would be approximately 20 Å apart.

Below we report experimental tests of the prediction that clusterin is a heparin-binding protein. We show that human clusterin binds heparin with a sub-micromolar affinity constant. The binding of heparin to clusterin is independent of the presence or absence of divalent cations but increases markedly at acidic pH. The α chain of clusterin was found to retain heparin-binding activity after being reduced and alkylated under denaturing conditions.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies. Clusterin-specific MAbs G7 (IgG1, κ) and 41D (IgG1, κ) have already been described (*24*, *25*). Rabbit IgG was prepared as in ref *26*. Clusterin was prepared from human serum by G7 immunoaffinity chromatography using a method based on ref *12*. Normal human serum was filtered through a glass-fiber filter, made 10 mM in EDTA and 0.5 mM in phenylmethylsulfonylfluoride, and then passed over a MAb G7−Sepharose column equilibrated in 0.01 M phosphate, 0.15 M NaCl, 3 mM NaN_3, pH 7.4 (PBS). Unbound protein was eluted with PBS, and the column was then washed with 1% (w/v) Triton X-100 in PBS to remove clusterin-bound lipoprotein and lipids. Bound clusterin was then eluted with 2 M guanidine HCl, 10 mM Tris, pH 7.4, and thereafter it was extensively dialyzed against PBS. Contaminating IgG was removed from these preparations by Protein G−Sepharose affinity chromatography. Bovine serum albumin (BSA), heat-denatured casein, and hen egg white lysozyme were all from Sigma-Aldrich, Castle Hill NSW, Australia.

Preparation of Biotinylated Heparin. Porcine heparin (Grade 1-A, sodium salt, M_r 1×10^4g/mol) was from Sigma-Aldrich. Native heparin was biotinylated by the method of ref *27*; heparin at 4 mg/mL in azide-free PBS was mixed with biotin-X-NHS (Calbiochem, San Diego) from a 10 mg/mL solution in DMSO to give a 1:10 w/w ratio of biotin-X-NHS to heparin. After a 1 h incubation at room temperature the solution was dialyzed against PBS and stored at 4 °C until use.

When heparin was biotinylated using the method shown in Figure 1 it was first dissolved to 10 mM in 2 M NaOH. Samples (2 mL) were made 30 mM in $NaBH_4$ (from a 0.3 M stock) and 1.9 M in epichlorohydrin, and the solution was made up to 10 mL with 2 M NaOH. This solution was incubated at room temperature for 24 h and then dialyzed against water. 1,6-Diaminohexane was then added to 0.1 M (from a 0.5 M stock in 0.1 M $NaHCO_3$, pH 8.5), and after a further 24 h incubation at room temperature the solution was dialyzed against 0.1 M $NaHCO_3$, pH 8.5. Biotin-X-NHS from a 10 mg/mL solution in DMSO was added to give a 1:1 (w/w) ratio to heparin. After a 3 h incubation at 37 °C the solution was dialyzed against water and freeze-dried, and the biotinylated heparin (b-heparin) was stored at 4 °C until use.

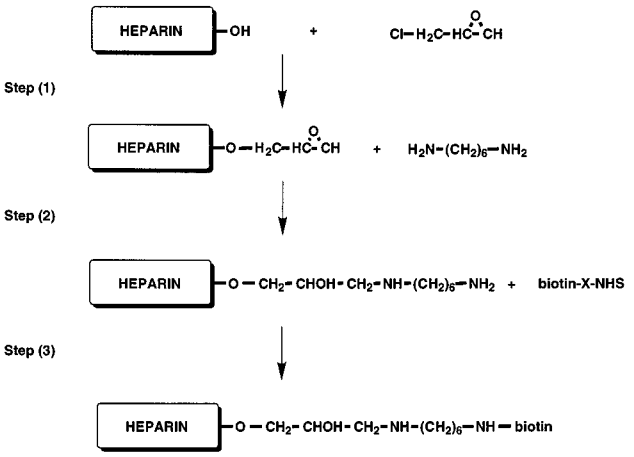

FIGURE 1: Reaction scheme for preparation of biotinylated heparin. Step (1), reaction of heparin with epichlorohydrin, yielding propylepoxyheparin; step (2), reaction of propylepoxyheparin with 1,6-diaminohexane, yielding activated heparin; step (3), reaction of activated heparin with biotin-X-NHS, yielding b-heparin.

The amino group content of heparin samples was determined using a method based on ref *28*. Heparin in 0.1 M $NaHCO_3$, pH 8.5, was made 0.12 mM in trinitrobenzenesulfonate (Sigma) and incubated in the dark for 3 h at room temperature. The absorbances of the solutions at 367 nm were measured, and the amino content of the samples was calculated assuming an extinction coefficient for trinitrophenyl−amino adducts of 1.1×10^4 M^{-1} cm^{-1} (*28*). The absorbance arising from spontaneous hydrolysis of trinitrobenzenesulfonate was corrected for using samples from which heparin was omitted.

ELISA Methods. Aliquots (50 μL) of clusterin (typically 20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) were applied to polystyrene ELISA trays (Disposable Products, Adelaide, Australia) and allowed to adsorb for 1 h at 37 °C. The wells were then blocked with 100 μL aliquots of azide-free PBS containing 1% (w/v) heat-denatured casein and 0.04% (w/v) thymol (HDC) for 30 min at 37 °C. b-heparin in 50 mM MES, 100 mM NaCl, 1% (w/v) BSA, 3 mM NaN_3, pH 6.0 (MES buffer) was next applied to the trays in 50 μL aliquots and incubated for 1 h at 37 °C. Bound b-heparin was detected using 50 μL aliquots of horseradish peroxidase-conjugated streptavidin (Boehringer-Mannheim Australia P/L, Castle Hill, Australia) diluted 1:2000 in azide-free MES buffer containing 0.04% (w/v) thymol with a 1 h incubation at 37 °C. The ELISA trays were washed 10 times with water between each of the above steps. The substrate solution used was 100 μL aliquots of 2.5 mg/mL *o*-phenylenediamine dihydrochloride in 0.05 M citrate, 0.1 M phosphate, pH 5, containing 0.03% (v/v) H_2O_2. The reaction was stopped using 50 μL aliquots of 2 M H_2SO_4, and the absorbance of the product formed was measured at 490 nm using a BioRad Model 450 plate reader.

Samples of diluted human serum that had been fractionated over heparin−Sepharose as below were analyzed for clusterin by ELISA. Aliquots were applied to ELISA trays as above at dilutions where there was a linear relationship between sample dilution and ELISA signal. The wells were blocked with HDC as above, and then bound clusterin was detected using tissue culture supernatant from G7 hybridoma cells as the source of primary antibody. Bound G7 antibody was detected using horseradish peroxidase-conjugated sheep anti-

mouse Ig antibody (Silenus Laboratories, Melbourne, Australia) diluted 1:2000 in HDC.

Immunoblotting Methods. In Western blotting procedures, clusterin which had been resolved on 10% SDS−PAGE gels under either reducing or nonreducing conditions was electrophoretically transferred to nitrocellulose (Gelman Sciences, Ann Arbor) using a Hoefer TE Series electrophoresis unit and 25 mM Tris, 192 mM glycine, 20% (v/v) methanol as transfer buffer. The nitrocellulose was then blocked by a 1 h incubation in HDC, and bound clusterin was detected using 41D hybridoma tissue culture supernatant as the primary antibody source and horseradish peroxidase-conjugated sheep anti-mouse Ig antibody diluted 1:2000 in HDC as the secondary antibody. The blots were developed using a substrate solution containing 1 volume 30 mg/mL 4-chloro-1-naphthol in methanol: 5 volumes azide-free PBS: 1 μL/ml 30% (w/v) hydrogen peroxide. In some experiments bound primary antibody was detected using ^{125}I-labeled sheep anti-mouse Ig antibody. In these cases the bound secondary antibody was visualized using a Molecular Dynamics phosphorimager and ImageQuant software. In dot-blot procedures, 10 μL aliquots of reduced and alkylated clusterin at concentrations between 1 and 10 μg/mL in 0.1 M $NaHCO_3$, pH 9.5, were applied to nitrocellulose and allowed to adsorb for 15 min at room temperature. The nitrocellulose was then blocked and probed using MAb 41D and horseradish peroxidase-conjugated sheep anti-mouse Ig antibody as described above.

Heparin−Sepharose Affinity Chromatography of Human Serum. Samples of human serum (0.2 mL) were diluted to 2 mL with either PBS or protein-free MES buffer. The samples were then applied to a 10 mL column of heparin−Sepharose (AMRAD Pharmacia Biotech, Melbourne, Australia) equilibrated with either PBS or MES buffer. Unbound protein was eluted with the equilibration buffer; the absorbance at 280 nm of the eluate was measured using an LKB Uvicord S column monitor. When the absorbance reached baseline, bound protein was eluted with 10 mM phosphate, 2 M NaCl, 3 mM NaN_3, pH 7.4. Aliquots of the pools of unbound and bound protein were diluted into PBS and analyzed for clusterin by ELISA and immunoblotting as described above.

Binding of Reduced and Alkylated Clusterin to Heparin−Sepharose. Clusterin was reduced and alkylated using a method based on ref *29*. Clusterin (1 mg) in PBS was made 6 M in guanidine HCl and incubated at 80 °C for 10 min. The solution was then made 10 mM in 2-mercaptoethanol and incubated at 80 °C for 15 min. It was then made 20 mM in iodoacetamide, incubated for a further 1 h at 80 °C, and then dialyzed against 10 mM phosphate, pH 6.5. Cleavage of the interchain disulfide bonds of clusterin was confirmed by SDS−PAGE. The reduced and alkylated clusterin was then applied to a heparin−Sepharose column, equilibrated in 10 mM phosphate, pH 6.5; the absorbance of the eluate was monitored as above. Unbound material was eluted with the same buffer, and then bound material was eluted with 10 mM phosphate, 1 M NaCl, pH 6.5. The bound fraction was concentrated by ultrafiltration and then subjected to six cycles of N-terminal sequencing. This was done by the Australian Proteome Analysis Facility (Macquarie University, Sydney) on a Hewlett-Packard G1000A Protein Sequencer using the standard protocol for that instrument.

Data Analysis. Where appropriate, data were subjected to nonlinear regression analysis using KaleidaGraph Version 3.0.5, Synergy Software, Reading, PA).

RESULTS

Development of a Novel Method for Biotinylation of Heparin. In preliminary experiments, heparin which had been biotinylated by the method of ref *27* was used in ELISA studies of the heparin-binding properties of clusterin. In this method, native heparin is treated with a biotin-*N*-hydroxysuccinimide ester; we found that biotinylated heparin (b-heparin) made in this way gave weak and variable signals in ELISA, regardless of whether clusterin or chicken lysozyme [an authentic heparin-binding protein (*27*)] was used as the target protein. We reasoned that this may have reflected the fact that the reaction chemistry of the method of ref *27* involves a reaction between the biotin-*N*-hydroxysuccinimide ester and free amino groups on heparin; typically most of the amino groups of the glucosamine residues of heparin are either acetylated or sulfated and are therefore unavailable for reaction with biotin-*N*-hydroxysuccinimide esters. Thus, the poor results of our preliminary experiments may have been a consequence of low levels of biotinylation of the heparin used.

Hence we developed a method, shown schematically in Figure 1, for increasing the number of free amino groups on heparin prior to biotinylation. In this method, epoxy groups are first introduced into heparin using epichlorohydrin. Heparin that has been activated in this way is then allowed to react with 1,6-diaminohexane, generating an amino derivative which can then be biotinylated using a biotin-*N*-hydroxysuccinimide ester. In a series of trial experiments, the effects of varying both the concentrations of epichlorohydrin and 1,6-diaminohexane as well as the effects of different reaction buffers and reaction times on biotinylation of heparin were studied, leading to the protocol for biotinylation of heparin described in Experimental Procedures. We confirmed that this procedure led to an increase in the amino group content of heparin using the trinitrobenzene sulfonate assay described in Experimental Procedures; native heparin had 0.7 ± 0.2 amino groups/mol while heparin which had been treated with epichlorohydrin and 1,6-diaminohexane had 15 ± 1 amino groups/mol (means $\pm$ standard deviations of triplicate determinations).

A comparison of the ELISA reactivities of b-heparin made in this way and b-heparin made by the method of ref *27*, using clusterin as the target protein, is shown in Figure 2A. There were dose-dependent increases in the ELISA signal with heparin concentration for both types of b-heparin. However, it is apparent that, when present at saturating levels, b-heparin made using the scheme shown in Figure 1 gives an approximately 10-fold greater ELISA signal than heparin made as in ref *27*. This observation, combined with the estimates of the amino group content of native heparin and heparin derivatized as in Figure 1, suggests that the poor ELISA signals obtained using b-heparin made as in ref *27* were a consequence of low levels of biotinylation of heparin. The observation that there was negligible binding of b-heparin made using the scheme shown in Figure 1 to rabbit

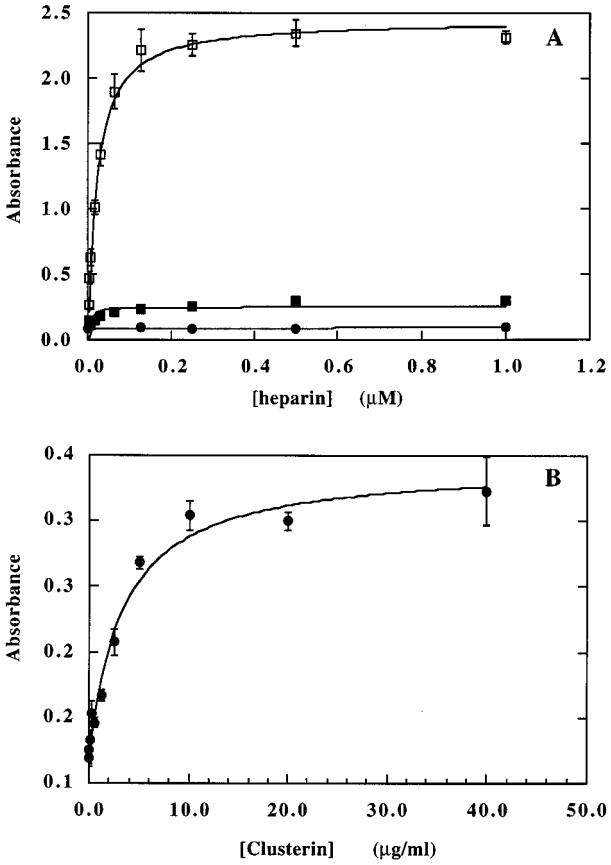

FIGURE 2: Binding of b-heparin to clusterin. (A) Comparison of different types of b-heparin. Clusterin (6 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was used to coat an ELISA tray, which was then blocked with HDC as described in Experimental Procedures. The binding of b-heparin made either by the method of Zou et al. (■) or heparin which had been activated as in Figure 1 (□) was then measured as in Experimental Procedures. The binding of heparin which had been activated as in Figure 1 to rabbit IgG, applied to an ELISA tray at 25 μg/mL (●) was measured in the same way. The data shown are means ± standard deviations of triplicate determinations; in some cases, the error bars are smaller than the data symbols. (B) Dependence of b-heparin binding on clusterin concentration. Clusterin at the indicated concentrations in 0.1 M $NaHCO_3$, pH 9.5, was used to coat an ELISA tray. The binding of b-heparin (0.3 μM) which had been activated as in Figure 1 was then measured as in Experimental Procedures. The data shown are means ± standard deviations of triplicate determinations.

IgG (Figure 2A) suggests that the increased signals obtained using this form of b-heparin did not reflect nonspecific binding to clusterin.

The data shown in Figure 2A imply that clusterin is a heparin-binding protein. To confirm this, we examined the relationship between the concentration of clusterin used to coat the ELISA trays and the ELISA signal arising from bound b-heparin. When this was done, the data in Figure 2B were obtained. The observation that there were dose-dependent increases in the ELISA signal from bound b-heparin with increasing concentrations of clusterin shows that clusterin is a heparin-binding protein.

Before embarking on a quantitative analysis of the heparin-binding properties of clusterin, we sought to obtain evidence that heparin biotinylated as in Figure 1 did not have compromised protein-binding activity. We did this by first determining the dose-dependence of the binding of b-heparin to lysozyme (Figure 3, solid symbols). These data were

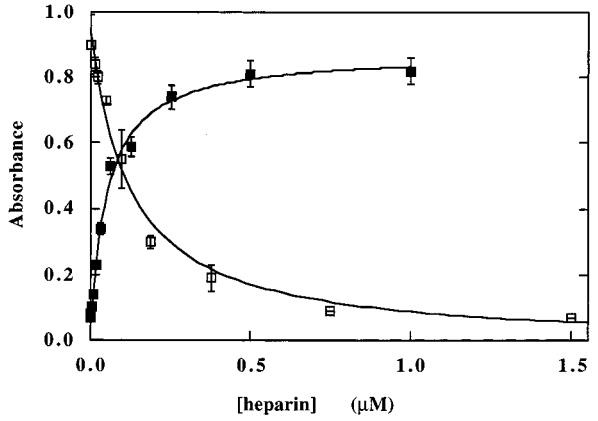

FIGURE 3: Binding of b-heparin to lysozyme. Lysozyme (20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was used to coat ELISA trays. The binding of b-heparin at the indicated concentrations (■) was then measured, and in a separate experiment, the effects of unlabeled heparin at the indicated concentrations (□) on the binding of b-heparin (0.3 μM) were determined, using the methods in Experimental Procedures in both cases. The data shown are means ± standard deviations of triplicate determinations; in some cases the error bars are smaller than the data symbols.

analyzed assuming that the binding of b-heparin to lysozyme is a noncooperative process. Equation 1 describes the dependence of the ELISA signal on the concentration of b-heparin for this case:

$$A = \frac{A_{max}[\text{b-heparin}]}{K + [\text{b-heparin}]} + \text{NS} \quad (1)$$

where A, A_{max}, [b-heparin], K, and NS represent, respectively, the absorbance obtained in ELISA, the absorbance at saturating levels of b-heparin, the concentration of b-heparin, the apparent dissociation constant for lysozyme−b-heparin complexes, and the contribution to the measured absorbance from nonspecifically bound b-heparin. Nonlinear regression of this equation onto the solid symbol data set shown in Figure 3 gave an estimate for K of 0.043 ± 0.005 μM. This is in good agreement with the value (0.04 μM) obtained by ref *27* in their study of the binding of b-heparin to lysozyme.

As an additional test of the protein-binding activity of the b-heparin, we used native heparin to inhibit the binding of b-heparin to lysozyme in competition experiments (Figure 3, open symbols). These data were analyzed using eq 2:

$$A = \frac{A_{max}}{(1 + [\text{heparin}]/K_i)} + \text{NS} \quad (2)$$

where [heparin] and K_i are the concentrations of native heparin and the apparent dissociation constant for lysozyme−native heparin complexes, respectively, with the other symbols being as in eq 1. Nonlinear regression of eq 2 onto the open symbols in Figure 3 gave an estimate for K_i of 0.11 ± 0.02 μM. The finding that this value was similar to the dissociation constant for b-heparin suggests that biotinylation of heparin as in Figure 1 does not compromise its protein-binding activity.

Quantitative Analysis of the Binding of b-Heparin to Clusterin in ELISA. To assess the apparent affinity of heparin for clusterin, experiments of the type shown in Figure 3 were carried out using clusterin as the target protein. In the first series of experiments, the dependence of the ELISA

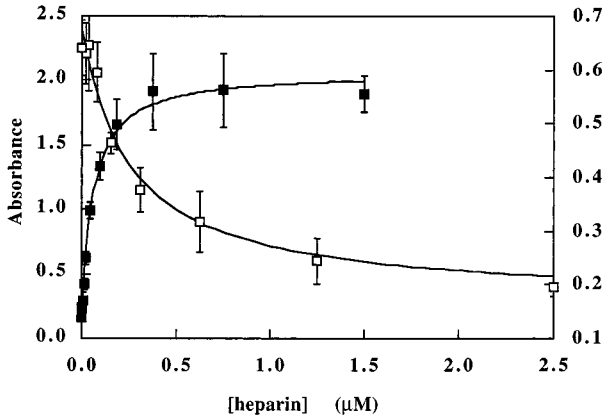

FIGURE 4: Concentration dependence of the binding of b-heparin to clusterin. Clusterin (20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was used to coat ELISA trays. The binding of b-heparin at the indicated concentrations (■) was then measured (absorbances shown on left ordinate). In a separate experiment, the effects of unlabeled heparin at the indicated concentrations (□) on the binding of b-heparin (0.3 μM) were determined (absorbances shown on right ordinate), using the methods in Experimental Procedures in both cases. The data shown are means ± standard deviations of triplicate determinations.

signal from bound b-heparin on the input concentration of b-heparin was established. The results of these (Figure 4, closed squares) show that the binding of b-heparin to clusterin was a saturable phenomenon. Nonlinear regression of eq 1 onto the b-heparin−clusterin binding data gave an estimate for the apparent dissociation constant for clusterin−b-heparin complexes of 0.061 ± 0.009 μM. Analysis of four independent experiments of this type gave a mean value (± standard deviation) for the apparent dissociation constant of 0.06 ± 0.02 μM. Native heparin inhibited the binding of b-heparin to clusterin in a dose-dependent manner (Figure 4, open squares). Nonlinear regression of eq 2 onto these data gave an estimate for the apparent dissociation constant for clusterin−heparin complexes of 0.25 ± 0.06 μM.

Dependence of the Binding of Heparin to Clusterin on Ca^{2+} and pH. The binding of heparin to a number of proteins has been shown to be modulated by calcium ions or other divalent cations. For example, the hemopexin-like domain of human gelatinase A binds to heparin in the presence of Ca^{2+} (*30*). The binding of heparin to human serum amyloid P component is highly Ca^{2+}-specific, in that no binding was observed in the presence of either Ba^{2+} or Mg^{2+} (*31*). In contrast, heparin has been shown to bind to human fibrinogen independently of the presence or absence of divalent cations (*32*). However, when the binding of b-heparin to clusterin was measured in the presence of either 1 mM $CaCl_2$ or 1 mM EDTA (Figure 5), no significant difference in the apparent affinity of heparin for clusterin was seen; nonlinear regression of eq 1 onto each of the data sets in Figure 5 gave estimates for K of 0.065 ± 0.019 μM and 0.091 ± 0.010 μM for the $CaCl_2$ and EDTA data sets, respectively.

In contrast, the binding of heparin to clusterin was found to vary with pH, being maximal under mildly acidic conditions (Figure 6). These data were analyzed assuming that the variation in the binding of heparin was a consequence of the ionization of a single residue on clusterin. This is described by eq 3:

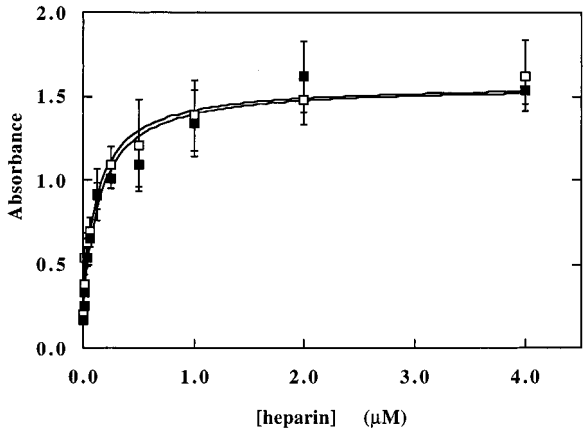

FIGURE 5: Dependence of the binding of b-heparin to clusterin on divalent cations. Clusterin (20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was used to coat ELISA trays. The binding of b-heparin at the indicated concentrations was measured in the presence of either 1 mM $CaCl_2$ (■) or 1 mM EDTA (□) using the methods in Experimental Procedures in both cases. The data shown are means ± standard deviations of triplicate determinations.

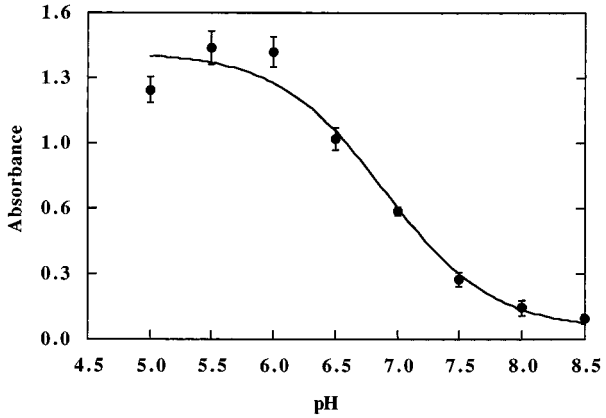

FIGURE 6: Dependence of the binding of b-heparin to clusterin on pH. Clusterin (20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was used to coat an ELISA tray. The binding of b-heparin (0.3 μM in 25 mM MES, 5 mM phosphate, 0.125 M NaCl, 10 mg/mL BSA, adjusted to the indicated pH values) was then measured; bound b-heparin was detected using horseradish peroxidase-conjugated streptavidin diluted 1:2000 into the above buffer at appropriate pH. The data shown are means ± standard deviations of triplicate determinations.

$$A = \frac{A_{low}[H^+] + A_{high}K}{[H^+] + K} \quad (3)$$

where A_{low}, A_{high}, and K represent the limiting absorbance at low pH, the limiting absorbance at high pH, and the dissociation constant for the ionizable residue, respectively. Nonlinear regression of eq 3 onto the data in Figure 6 gave an estimate for K of (1.3 ± 0.4) × 10^{-7} M, corresponding to a pK_a of 6.9.

The conclusion that clusterin binds preferentially to heparin under mildly acidic conditions was corroborated by the results of affinity purification of clusterin from dilute human serum using heparin−Sepharose (Figure 7). ELISA analysis of the clusterin in dilute human serum which had been passed over heparin−Sepharose equilibrated with PBS, pH 7.4, showed that only trace amounts of clusterin were retained by this affinity matrix (Figure 7A, open bars). Conversely, when dilute serum was adjusted to pH 6.0 and then passed over heparin−Sepharose equilibrated with MES buffer, pH

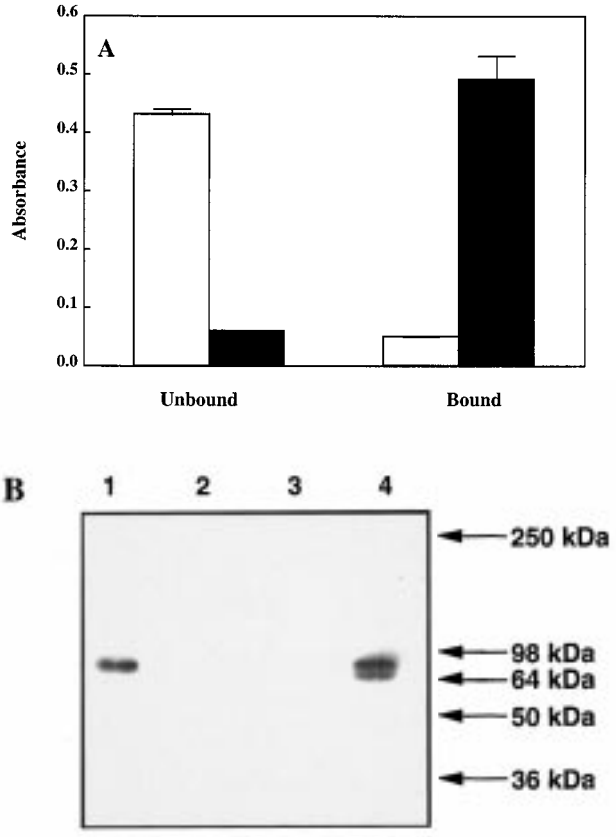

FIGURE 7: Fractionation of human serum on heparin−Sepharose at pH 7.4 and 6.5. (A) ELISA analysis. Samples of diluted human serum were fractionated on heparin−Sepharose at either pH 7.4 (open bars) or 6.0 (closed bars) as described in Experimental Procedures. The clusterin content of the wash-through and bound fractions (both diluted 1:400) was assessed by ELISA, as described in Experimental Procedures. The data shown are means ± standard deviations of triplicate deteminations. B. Immunoblotting analysis. Aliquots of the bound and unbound material were subjected to SDS−PAGE under nonreducing conditions, transferred to nitrocellulose and then probed for clusterin using MAb 41D and ^{125}I-labeled sheep anti-mouse Ig antibody as described in Experimental Procedures. The samples were as follows: lane 1, bound fraction, pH 6.0; lane 2, wash-through fraction, pH 6.0; lane 3, bound fraction, pH 7.4; lane 4, wash-through fraction, pH 7.4. The migration positions of molecular weight standards are indicated by arrows.

6.0, the bulk of the clusterin was retained by the column and could be eluted with PBS containing 2 M NaCl (Figure 7A, closed bars). When we analyzed aliquots of the wash-through and bound fractions for clusterin by immunoblotting (Figure 7B), the results confirmed the conclusions drawn from the ELISA analyses. Clusterin (at its expected size of approximately 80 kDa) was detectable only in the wash-through fraction of heparin−Sepharose columns which had been equilibrated at pH 7.4 (Figure 7B, lane 4). Conversely, clusterin was only detectable in the bound fraction of heparin−Sepharose columns which had been equilibrated at pH 6.0 (Figure 7B, lane 1).

Binding of Reduced and Alkylated Clusterin to Heparin−Sepharose. The data presented above show that clusterin can bind to heparin under nondenaturing conditions. The heparin binding site(s) of clusterin could be composed either of amino acid residues which are contiguous in the sequence of the protein (sequential heparin-binding sites) or of residues which are distant in the sequence of the protein, but which are brought into physical proximity by the three-dimensional

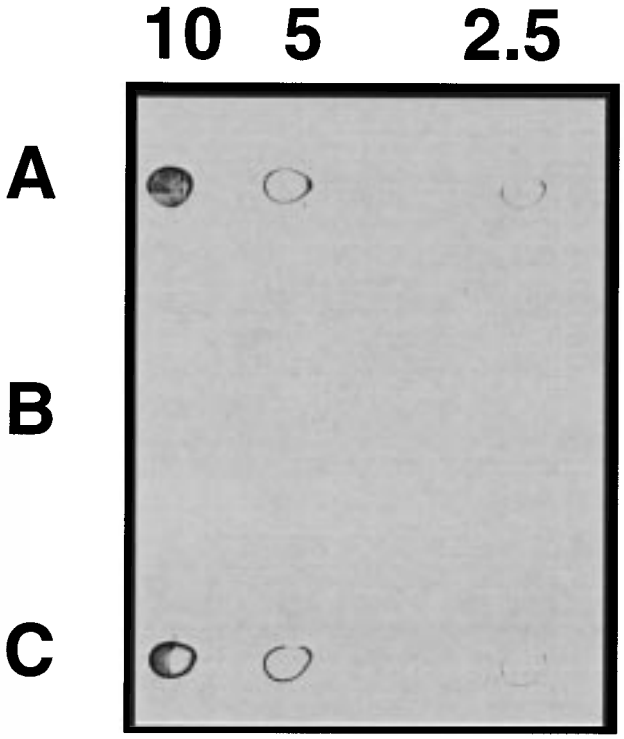

FIGURE 8: Dot-blot analysis of reduced and alkylated clusterin fractionated on heparin−Sepharose. Aliquots (10 μL) of reduced and alkylated clusterin at the indicated concentrations in μg/mL were applied to nitrocellulose which was then probed with MAb 41D as described in Experimental Procedures. The samples were as follows: (A) reduced and alkylated clusterin prior to fractionation on heparin−Sepharose, (B) the fraction which did not bind to the column, and (C) the fraction which bound to the column.

structure of native clusterin (conformational heparin-binding sites). In an attempt to assess whether clusterin contains sequential rather than conformational heparin-binding sites, samples of clusterin which had been reduced and alkylated under denaturing conditions were subjected to affinity chromatography over heparin−Sepharose. When this was done it was found that, as judged by the absorbance at 280 nm of the eluate, approximately half of the reduced and alkylated clusterin was present in the break-through fraction, while the remainder bound to the column and could be eluted with 0.01 M phosphate, 0.5 M NaCl, pH 7.4. Analyses of the break-though and bound material by dot-blots, using MAb 41D as the detection antibody, showed that this MAb reacted with the unfractionated reduced and alkylated clusterin and with the bound material but not with the break-through fraction (Figure 8). As we have established that MAb 41D detects only one of the polypeptide chains of clusterin in Western blotting experiments after the protein has been subjected to SDS−PAGE under reducing conditions (data not shown), the data shown in Figure 8 imply that one of the polypeptide chains of clusterin contains a heparin-binding site which is retained after the protein has been reduced and alkylated under denaturing conditions. To identify this chain, samples of the material which bound to heparin−Sepharose were subjected to six cycles of N-terminal sequencing. Only one amino acid was detected at each cycle, and the sequence obtained was glu-gln-thr-val-ser-glu. This sequence is identical to that of the first six residues of the α chain of processed human clusterin (*2*, *15*, *33*). These data therefore imply that the α chain of clusterin

contains a heparin-binding site which is retained after the protein has been reduced and alkylated under denaturing conditions.

DISCUSSION

There are four novel aspects of the data presented above: development of a new method for biotinylation of heparin, use of heparin which had been biotinylated in this way to demonstrate that clusterin is a heparin-binding protein, demonstration by ELISA and affinity chromatography that clusterin binds preferentially to heparin at low pH, and demonstration that the α chain of clusterin contains a heparin-binding site which is retained following reduction and alkylation of the interchain disulfide bonds of clusterin.

A number of different strategies have been used to prepare b-heparin. In some cases (e.g., *27*, *34*) native heparin was biotinylated using amino group-reactive reagents. In our hands (Figure 2A), b-heparin made in this way gave relatively poor signals in ELISA, presumably as a consequence of the limited number of amino groups in native heparin available for biotinylation. This potential problem has been recognized by other workers, who have derivatized heparin to increase its amino group content prior to biotinylation. For example, heparin has been activated with cyanogen bromide and then reacted it with 1,6-diaminohexane prior to biotinylation (*35*). Biotinylated heparin made in this way was used successfully in studies of the heparin-binding properties of tenascin-C (*36*). This strategy is similar to that used in this work, although the fact that epichlorohydrin is less toxic than cyanogen bromide may be an advantage of our approach. A different approach was used by Stearns et al. (*37*) who introduced amino groups into heparin using 3-bromopropylamine hydrobromide. They found that (depending on the target protein) there was up to an order of magnitude gain in sensitivity in dot-blot assays using b-heparin made from derivatized heparin compared to native heparin.

Although we have only used the procedure outlined in Figure 1 to prepare biotinylated heparin, in principle it could be used to prepare other labeled forms of proteoglycans. For example, treatment of proteoglycans which had been derivatized as in Figure 1 with fluorescein isothiocyanate could be used to prepare fluorescent derivatives for use in flow cytometry or histochemistry experiments.

Our analysis of the binding of heparin to clusterin shows that it is characterized by an apparent affinity constant of 0.06 ± 0.02 μM (Figure 4). This value is comparable to that (0.02 μM) reported for the binding of heparin to antithrombin (*38*) and falls in the midrange of reported affinity constants for heparin−protein interactions. These values range from 0.3 nM for heparin binding to the Alzheimer's disease amyloid precursor protein (*39*) to 4 μM for binding of heparin to low-density lipoproteins (*40*). Although we have shown that the α chain of clusterin contains a sequential heparin binding site (Figure 8) we cannot yet rule out the possibility that the predicted β chain heparin binding sites of clusterin (residues 402−408 and 420−427) contribute to its heparin-binding activity but are dependent on the conformational integrity of the protein, being lost following reduction and alkylation of the interchain disulfide bonds of clusterin under denaturing conditions.

The physiological significance of the ability of clusterin to bind heparin is not known. Increased clusterin expression is associated with a number of models of tissue damage or re-modeling, including regression of the rat prostate following androgen ablation, nephrotoxic or hydrostatic pressure kidney damage, neurodegenerative conditions, and myocardial infarction (reviewed in ref *41*). This has led to the suggestion that clusterin may serve as a scavenger for potentially deleterious hydrophobic molecules released at such sites (*42*). It is known that mast cells, the primary cellular source of heparin, can be chemotactically recruited to sites of tissue damage or repair, where they release heparin-containing granules (reviewed in ref *43*). We speculate that the ability of clusterin to bind to heparin released at such sites may serve to increase the local clusterin concentration and/or to modulate the ability of clusterin to remove hydrophobic molecules and other cellular debris. There is a precedent for this hypothesis; the binding of heparin to basic fibroblast growth factor-2 is known to potentiate its binding to cognate receptors (e.g., ref *44*).

Our finding that the binding of clusterin to heparin is enhanced at mildly acidic pH (Figures 6, 7) may be an example of a general phenomenon for clusterin-binding ligands. It has been reported that the binding of IgG, complement protein C9, and the β-amyloid peptide of Alzheimer's disease to clusterin are all enhanced at pH 6.0 compared to 7.5.[2] As heparin, IgG, C9, and β-amyloid peptide are structurally disparate molecules, it is unlikely that their ability to bind preferentially to clusterin at low pH reflects pH-induced structural changes in their clusterin-binding sites. A more plausible explanation may be that there are pH-induced changes in the structure of clusterin which lead to an enhancement of its ability to bind these ligands.[3] The preferential binding of clusterin to heparin under mildly acidic conditions may also be relevant to the above speculative hypothesis for the physiological importance of this interaction. Local acidosis may occur at sites of tissue damage or inflammation, with the pH at such sites falling to as low as 6 (*45*, *46*). In some cases the local acidosis may be prolonged; a local pH of 6.09 ± 0.19 was recorded in damaged rat brain tissue 7 h after experimental occlusion of the middle cerebral artery (*46*). It is thus possible that local acidosis at sites of tissue damage may persist long enough to allow augmentation of the clusterin−heparin interaction, hence facilitating the putative scavenger function of clusterin.

ACKNOWLEDGMENT

This research has been facilitated by access to the Australian Proteome Analysis Facility, established under the Australian Government's Major National Research Facilities Program. We are grateful to Dr. Brendan Murphy (St Vincent's Hospital, Melbourne, Australia) for his gift of the G7 hybridoma cell line and to the NSW Red Cross Blood

[2] J. Lakins and M. Tenniswood, data presented at the third International Clusterin Workshop, Villars sur Ollon, Switzerland, January 1997.

[3] We have obtained evidence for pH-dependent changes in the structure of clusterin from fluorescence and analytical ultracentrifuge experiments (G. J. Pankhurst, T. T. Hochgrebe, and S. B. Easterbrook-Smith, unpublished observations).

Bank for supplying us with human serum. We thank Jon Lakins for helpful discussions of the pH-dependent ligand binding properties of clusterin and Dr. Mark Wilson for constructive comments.

REFERENCES

1. Blaschuk, O., Burdzy, K., and Fritz, I. B. (1983) *J. Biol. Chem. 258*, 7714−7720.
2. de Silva, H. V., Harmony, J. A. K., Stuart, W. D., Gil, C. M., and Robbins, J. (1990) *Biochemistry 29*, 5380−5389.
3. Watts, M. J., Danker, J. R., and Morgan, E. P. (1990) *Biochem. J. 265*, 471−477.
4. Kounnas, M. Z., Loukinova, E. B., Stefansson, S., Harmony, J. A. K., Brewer, B. H., Strickland, D. K., and Argraves, W. S. (1995) *J. Biol. Chem. 270*, 13070−13075.
5. Reddy, K. B., Jin, G., Karode, M. C., Harmony, J. A. K., and Howe, P. H. (1996) *Biochemistry 35*, 6157−6163.
6. Reddy, K. B., Karode, M. C., Harmony, J. A. K., and Howe, P. H. (1996) *Biochemistry 35*, 309−314.
7. Harmony, J. A. K. (1995) in *Clusterin: Role in Vertebrate Development, Function and Development* (Harmony, J. A. K., Ed.) pp 1−188, R. G. Landes Co., Austin, TX.
8. Tschopp, J. A., Chonn, A., Hertig, S., and French, L. E. (1993) *J. Immunol. 151*, 2159−2165.
9. McDonald, J. F., and Nelsesteun, G. L. (1997) *Biochemistry 36*, 7464−7473.
10. Jenne, D. E., Lowin, B., Peitsch, M. C., Bottcher, A., Schmitz, G., and Tschopp, J. (1991) *J. Biol. Chem. 266*, 11030−11036.
11. Wilson, M. R., Roeth, P. J., and Easterbrook-Smith, S. B. (1991) *Biochem. Biophys. Res. Commun. 177*, 985−990.
12. Wilson, M. R., and Easterbrook-Smith, S. B. (1992) *Biochim. Biophys. Acta 1159*, 319−326.
13. Ghiso, J., Matsubara, E., Koudinov, A., Choi-Miura, N. H., Tomita, M., Wisniewski, T., and Frangione, B. (1993) *Biochem. J. 293*, 27−30.
14. Akesson, P., Sjoholm, A. G., and Bjorck, L. (1996) *J. Biol. Chem. 271*, 1081−1088.
15. Kirszbaum, L., Sharpe, B., Murphy, B. F., d'Apice, A. J., Classon, B., Hudson, P. J., and Walker, I. D. (1989) *EMBO J. 8*, 711−718.
16. French, L. E., Chonn, A., Ducrest, D., Baumann, B., Belin, D., Wohlwend, A., Kiss, J. Z., Sappino, A. P., Tschopp, J., and Schifferli, J. A. (1993) *J. Cell Biol. 122*, 1119−1130.
17. Tsuruta, J. K., Wong, K., Fritz, I. B., and Griswold, M. D. (1990) *Biochem. J. 268*, 571−578.
18. Jordan-Starck, T. C., Witte, D. P., Aronow, B. J., and Harmony, J. A. K. (1990) *Curr. Opin. Lipidol. 3*, 75−85.
19. Burkey, B. F., de Silva, H. V., and Harmony, J. A. K. (1991) *J. Lipid Res. 32*, 1039−1048.
20. Roeth, P. J., and Easterbrook-Smith, S. B. (1996) *Biochim. Biophys. Acta 1297*, 159−66.
21. Cardin, A. D., and Weintraub, H. J. R. (1989) *Arteriosclerosis 9*, 21−32.
22. Margalit, H., Fischer, N., and Ben-Sasson, S. A. (1993) *J. Biol. Chem. 268*, 19228−19231.
23. Rost, B., Sander, C., and Schneider, R. (1994) *Comput. Appl. Biosci. 10*, 53−60.
24. Murphy, B. F., Kirzbaum, L., Walker, I. D., and d'Apice, A. J. (1988) *J. Clin. Invest. 81*, 1858−1864.
25. Humphreys, D., Hochgrebe, T. T., Easterbrook-Smith, S. B., Tenniswood, M. P., and Wilson, M. R. (1997) *Biochemistry 36*, 15233−15243.
26. Easterbrook-Smith, S. B., Vandenberg, R. J., and Alden, J. R. (1988) *Mol. Immunol. 25*, 1331−1337.
27. Zou, S., Magura, C., and Hurley, W. (1992) *Comp. Biochem. Physiol. 103B*, 889−895.
28. Glazer, A. N., DeLange, R. J., and Sigman, D. S. (1976) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., and Work, E., Eds.) pp 76−78, North-Holland Publishing Company, Amsterdam, The Netherlands.
29. Harlow, E., and Lane, D. (1988) In *Antibodies: A Laboratory Manual*, pp 65−68, Cold Spring Harbor Laboratory Press, New York.
30. Wallon, U. M., and Overall, C. M. (1997) *J. Biol. Chem. 272*, 7473−7481.
31. Li, X. A., Hatanaka, K., Guo, L., Kitamura, Y., and Yamamoto, A. (1994) *Biochim. Biophys. Acta 120*, 143−148.
32. Mohri, H., and Ohkubo, T. (1993) *Arch. Biochem. Biophys. 303*, 27−31.
33. Jenne, D. E., and Tschopp, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7123−7127.
34. Foxall, C., Holme, K. R., Liang, W., and Wei, Z. (1995) *Anal. Biochem. 231*, 366−373.
35. Debbage, P. L., Lane, W., Helmann, T., and Gabius, H.-J. (1988) *J. Histochem. Cytochem. 36*, 1097−1102.
36. Weber, P., Zimmermann, D. R., Winterhalter, K. H., and Vaughan, L. (1995) *J. Biol. Chem. 270*, 4619−4623.
37. Stearns, N. A., Prigent-Richard, S., Letourneur, D., and Castellot, J. J. (1997) *Anal. Biochem. 247*, 348−356.
38. Barrow, R. T., Parker, E. T., Krishnaswamy, S., and Lolla, R. P. (1994) *J. Biol. Chem. 269*, 26796−26800.
39. Multhaup, G., Mechler, H., and Masters, C. L (1995) *J. Mol. Recognit. 8*, 247−257.
40. Gigli, M., Consonni, A., Ghiselli, G., Rizzo, V., Naggi, A., and Torri, G. (1992) *Biochemistry 31*, 5996−6003.
41. Jenne, D. E., and Tschopp, J. (1992) *Trends Biochem. Sci. 17*, 154−159.
42. James, R. W. (1995) in *Clusterin: Role in Vertebrate Development, Function and Development* (Harmony, J. A. K., Ed.), pp 13−32, R. G. Landes Co., Austin, TX.
43. Hamawy, M. M., Mergenhan, S. E., and Siraganian, R. P. (1994) *Immunol. Today 15*, 62−66.
44. Moy, F. J., Safran, M., Seddon, A. P., Kitchen, D., Bohlen, P., Aviezer, D., Yayon, A., and Powers, R. (1997) *Biochemistry 36*, 4782−4791.
45. Punnia-Moorthy, A. (1988) *Br. J. Anaesth. 61*, 154−159.
46. Back, T., Hoehn-Berlage, M., Kohno, K., and Hossmann, K. A. (1994) *Stroke 25*, 494−500.

BI972367V