Decorin is a Zn^{2+} Metalloprotein

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SUMMARY

Decorin is ubiquitously distributed in the extracellular matrix of mammals and a member of the proteoglycan family characterized by a core protein dominated by Leucine Rich Repeat motifs. We here demonstrate that decorin extracted from bovine tissues under denaturing conditions or produced in recombinant "native" form by cultured mammalian cells, has a high affinity for Zn^{2+}. Binding of Zn^{2+} to decorin is demonstrated by Zn^{2+} chelating chromatography and equilibrium dialyses. The Zn^{2+} binding sites are localized to the N-terminal domain of the core protein that contains 4 Cys residues in the spacing reminiscent of a Zn finger. A recombinant 41 amino acid long peptide representing the N-terminal domain of decorin has full Zn^{2+} binding activity and binds two Zn^{2+} ions with an average K_D of $3x10^{-7}M$. Biglycan, a proteoglycan that is structurally closely related to decorin contains a similar high affinity Zn^{2+} binding segment, whereas the structurally more distantly related proteoglycans, epiphycan and osteoglycin, did not bind Zn^{2+} with high affinity.

INTRODUCTION

Decorin, a small chondroitin/dermatan sulfate proteoglycan, is found in the extracellular matrix of a variety of tissues such as skin (1-3), cartilage (4-5), and bone (6-7). This proteoglycan is composed of a 40 kDa core protein and one glycosaminoglycan chain attached to a serine residue in the N-terminal part of the protein. The decorin core protein is dominated by a central region composed of ten Leucine Rich Repeat units. Each unit contains 21-26 amino acid residues and is proposed to adopt a characteristic α-helix/β-sheet folding pattern (8-9). The C- and N-terminal regions of the core protein are believed to form globular structures stabilized by disulfide bonds between sets of cysteine residues. Several proteoglycans have core proteins of similar size and structural organization. These related molecules are considered to form a family called Small Leucine Rich Proteoglycans (SLRP) (9-10). The family of SLRPs include decorin, biglycan, and epiphycan, all of which contain chondroitin/dermatan sulfate chains attached to the N-terminal domain of the core protein and fibromodulin, lumican, keratocan, PRELP, and osteoglycin which often have keratan sulfate linked to asparagine residues in the central region of the core protein. The extracellular matrix glycoprotein chondroadherin has a structural organization similar to the core proteins of the SLRPs but has not been shown to be substituted with glycosaminoglycan chains (11). The biological importance of the different SLRPs is unclear. *In vitro* binding studies have shown that decorin, biglycan, and fibromodulin can interact with several types of collagen (12-16) and different SLRPs are believed to be important regulators of collagen fibrillogenesis. In support of this hypothesis, a decorin deficient mouse was found to have fragile skin with an abnormal organization of collagen fibers (17). The phenotype appears to be largely restricted to the skin, perhaps suggesting that other SLRPs have similar functions and may fulfill this role in other collagenous tissues. In fact, a lumican deficient mouse also exhibited abnormal collagen fibers both in the skin and in the cornea (18).

Decorin may also affect the production of other extracellular matrix components by regulating the activity of TGF-ß (19-20). Additionally, decorin can modulate the interactions of

matrix molecules such as fibronectin with cells (21-23). These observations suggest that decorin and perhaps other SLRPs regulate at several levels the production and assembly of the extracellular matrix and hence the remodeling of connective tissue.

Zinc, a divalent cation, is one of the essential trace elements for eukaryotic organisms. Zinc ions plays a key role in biological processes by being directly involved in enzyme catalysis or by binding to specific sites in a protein to stabilize the conformations which are of importance to the function of the protein (24-25). In the extracellular matrix, zinc is required for the activity of matrix metalloproteases which are responsible for the degradation of structural extracellular matrix components (26). Breakdown and remodeling of the ECM occur in normal embryo development, wound healing, and many pathological processes such as cancer, arthritis, and osteoporosis (26-27). Structural extracellular matrix molecules such as laminin (29), link protein (30), nidogen (31), and COMP (32) have been shown to bind zinc ions. The Zn^{2+} binding sites have been located to subdomains of some of these proteins (31,33).

In a previous study, biglycan was shown to self associate in the presence of zinc ions and shown to bind Zn^{2+}, Ni^{2+}, and Cu^{2+} in metal chelate affinity chromatography studies (34). The binding affinity and binding domain, however, have not been reported. In this study, we demonstrate that decorin is a metalloprotein and binds two zinc ions per core protein with an average K_D of ~1μM. The Zn^{2+} binding domain is mapped to the N-terminal region in the decorin core protein and a short (41 amino acid long) peptide is shown to have full Zn^{2+} binding activity. Furthermore, the Zn^{2+} binding activity of the corresponding segments in the structurally related core proteins of biglycan, epiphycan, and osteoglycin are analyzed.

EXPERIMENTAL PROCEDURE

Preparation of Decorin Proteoglycans Decorin was extracted and purified from bovine skin under denaturing conditions as described previously (3). Intact recombinant decorin was produced in HT1080 cells using a vaccinia virus based expression system. The recombinant virus construct contains a segment encoding a N-terminal polyhistidine tag, a Factor Xa cleavage site and the mature human decorin core protein starting with an Asp residue found at position #31 of the full length human sequence (35). The polyhistidine tag in the recombinant protein allows for its efficient purification by Ni^{2+} chelating chromatography. The resulting decorin preparation contains a mixture of proteoglycan and core protein forms. The details of this system have been described earlier (35-36). For the studies described here, the polyhistidine tag was removed from the recombinant decorin by digestion with Factor Xa (Pierce, Rockford, IL) followed by reapplication of the digestion mixture to a Ni^{2+} charged column. The final product of cleaved recombinant decorin was collected in the "flow-through" fractions whereas the polyhistidine tag and uncleaved protein were retained on the column.

Recombinant Decorin Core Protein Fragments A series of recombinant decorin core protein fragments were produced in *E. coli.* A mouse embryo cDNA library (Clonetech, Palo Alto, CA) was used as a template along with appropriate primers (Table 1A) to PCR amplify the decorin cDNA segments. Conditions for PCR were 94°C/7 min, followed by 35 cycles involving 94°C/1 min, 50°C/2 min, and 72°C/3 min. The individual segments were purified, cleaved with appropriate restriction enzymes and ligated into the pMAL-p2 expression vector (New England Biolabs, Beverly, MA) which contain a segment encoding the maltose binding protein (MBP) at the 5' end of the expression cassette. The cDNA fragments (MD and MD3) generated with the DCNR2 primer which contains a EcoRI site at 3' end, were first subcloned into the pBluscript SK+/- vector (Stratagene, La Jolla, CA) through BamHI and EcoRI sites. Subsequently, the MD/pBluscript and MD3/pBluscript constructs were digested with BamHI and HindIII and then ligated into the pMAL-p2 expression vector. To produce MBP as a control protein without any

segment of decorin fused to it, a modified pMAL-p2 vector containing a stop codon and a short polylinker was constructed. The complementary oligonucleotides, MBPF (5'-gatcctgatctagagcatgcctgca-3') and MBPR (5'-ggcatgctctagatcag-3') were obtained and allowed to form a double stranded oligonucleotide containing a cohesive BamHI site at the 5'-end followed by a stop codon, XbaI, SphI and PstI sites. The original pMAL-p2 vector digested with BamHI and PstI was then ligated with that double stranded oligonucleotide. The newly introduced SphI site which is not present in the original pMAL-p2 vector was used in a restriction enzyme digestion screen to isolate clones carrying the engineered plasmid. The sequences of all decorin/pMAL-p2 constructs isolated from selected clones were confirmed by DNA sequencing (Department of Veterinary Pathobiology, Texas A&M University, College Station, TX).

Expression and Purification of Recombinant Decorin Core Protein Subdomains

Previously, a method developed for the production of full length bovine decorin core protein using a pMAL expression system has been reported (37). This procedure involved solubilization and refolding of protein recovered from inclusion bodies. In our case, we obtained significant amounts of soluble recombinant protein from all constructs and we did not attempt to solubilize protein from inclusion bodies.

The expression vector constructs were used to transform the *E. coli.* strain TB1. The procedure adopted for the expression and purification of the MBP-decorin fusion protein was based on the manual provided by New England Biolabs. In brief, a flask containing 950 ml of Lennox LB (Sigma, St. Louis, MO) was inoculated with 50 ml of a TB1/MBP-decorin overnight culture and grown on a platform shaker for 3~4 h at 37°C when the culture reached an OD_{600} of 0.6-0.8. Expression of fusion protein was induced by adding IPTG (Gibco BRL, Gaithersburg, MD) to the culture at a final concentration of 0.2mM and incubation was continued for another 3 h. The bacterial cells were harvested by centrifugation at 4500 rpm for 20 min. The cell pellet was resuspended in Buffer A (20mM Tris, 200mM NaCl, pH = 8.0) to a final volume of 10 ml/L of culture and frozen at -80°C for a minimum of 18 h. To purify the fusion proteins, the cells were thawed and lysed using a french press. The lysate was cleared by centrifugation at 40,000 rpm for

20 min and the supernatant was applied to a 5 ml amylose affinity column (New England Biolabs, Beverly, MA) equilibrated with 25 ml of Buffer A. The column was washed with 50 ml of Buffer A and the MBP-decorin fusion protein was eluted by Buffer A containing 10 mM maltose. The purity of the fusion protein was >70% as judged by SDS-PAGE and the yield was 5-10 mg/L of culture. To improve the purity of fusion proteins, an additional purification step was required. Ion exchange chromatography was used for further purifying MBP-MD2 and MBP-MD3 protein. The proteins were dialyzed into a buffer composed of 25 mM NaCl, 20 mM Tris, pH = 8.0 and applied to a column of Q-sepharose (Pharmacia Biotech, Piscataway, NJ) equilibrated with the same buffer. The protein was subsequently eluted with a NaCl gradient (25-500 mM) in 20 mM Tris, pH = 8.0. Metal ion chelating chromatography on iminodiacetic acid immobilized Sepharose 6B resin (Sigma, St. Louis, MO) charged with Zn^{2+} was used to further purify MBP-MD, MBP-MD1, and MBP-MD4. The procedure used for charging the column with Zn^{2+} ions is described in the following section. The purified recombinant proteins were analyzed on SDS-PAGE and Western blot using two polyclonal antibodies that were raised against synthetic peptides corresponding to the amino acid residues 31-47 and 309-326 of the mouse decorin core proteins respectively (Manuscript in preparation). The protein concentrations were determined based on the absorbance at A_{280} and the calculated molar extinction coefficient of the different protein constructs (38).

Production and Purification of a N-terminal Decorin Peptide (MD4). To produce large quantity of MD4 peptide, we used a pGEX-2T expression vector (Pharmacia, Piscataway, NJ). This MD4/pGEX-2T construct encodes glutathione S-transferase (GST) followed a thrombin cleavage site and the MD4 fragment. The cDNA fragment encoding MD4 was obtained by PCR using the mouse embryo cDNA library and primers DCNF9 and DCNR19 as described above (Table 1A). The resulting PCR fragment was cleaved by BamHI and PstI, purified, and subcloned into pBluscript. The isolated MD4/pBluscript plasmid was digested with BamHI and EcoRI and ligated into the pGEX-2T expression vector. The recombinant MD4/pGEX-2T plasmid was then transformed into the *E. coli.* strain, BL-21 (Stratagene, La Jolla, CA). An isolate clone containing the MD4/pGEX-2T plasmid was used after the plasmid had been analyzed by restriction enzyme

digestion and sequencing. To express the GST-MD4 fusion protein, flasks containing 950 ml of Lennox LB were inoculated with 15 ml of a BL21/GST-MD4 overnight culture and incubated for 3-4 h when the culture reached an A_{600} of 0.4-0.5. Recombinant protein expression was induced by adding IPTG to the culture at a final concentration of 0.2 mM. Bacterial cells were harvested 3 h later by centrifugation at 4500 rpm for 20 min. The cell pellet was resuspended in PBS (8.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 150 mM NaCl pH = 7.4), supplemented with 5 mM EDTA and adjusting the pH = 7.5 to a final volume of 10 ml/L of culture and frozen at -80°C for a minimum of 18 h. The cells were thawed and lysed using a french press. One ml of 10% Triton X-100 (Sigma, St. Louis, MO) was added to the cell lysate and mixed until homogeneous. The cell homogenate was centrifuged and filtered through a 0.45µ membrane to remove cell debris.

The GST-MD4 fusion protein present in the filtered supernatant was purified by affinity chromatography on a 10 ml column of Glutathione-agarose (Sigma, St. Louis, MO) equilibrated with 50 ml of Buffer B (PBS with 1 mM EDTA, pH = 7.5). The column was washed with 75 ml of Buffer B and the GST-MD4 protein was eluted from the column with 20 ml Buffer C (50 mM Tris, 10 mM glutathione, pH = 7.5). The purity of GST-MD4 in the eluate was >90%, as judged by SDS-PAGE and the yield was 25-30 mg/L of culture.

To isolate the MD4 peptide the fusion protein was cleaved with thrombin. The pH and the concentration of NaCl of the eluant containing GST-MD4 was adjusted to pH = 8.3 [NaCl] = 0.15 M using 1 M NaOH and 3 M NaCl stock solutions. Bovine thrombin (Sigma, St. Louis, MO) was added to give a 1/40 (w/w) enzyme/substrate ratio. The digestion was allowed to proceed overnight at room temperature. Subsequently, ß-mercaptoethanol was added to the incubation mixture to a final concentration of 1% (v/v) and the solution was adjusted to pH = 9.5 by addition of 1 M NaOH. The solution was incubated at 37°C for 30 min and filtered through a 0.45µ membrane to remove any particulate material. The MD4 peptide was purified on a Waters 25x200 mm RCM semi-preparative C18 HPLC column and eluted with a gradient of 28% to 36% solvent B (95% Acetonitrile, 5% H_2O, 0.1% TFA) in solvent A (95% H_2O, 5% Acetonitrile, 0.1% TFA) over 10 min at a flow rate of 25 ml min^{-1}.

The purity of the peptide was monitored by running a three layer Tricine-SDS-PAGE gel consisting of a 15% acrylamid slab, 10% acrylamid spacer, and a 3.5% acrylamid stacker (39). The identity of the peptide was confirmed by Matrix Assisted Laser Desorption Ionization-mass spectroscopy (MALDI-MS) (The Center for Analytical Chemistry, University of Texas in Houston, Houston, TX).

Biglycan Preparations Biglycan isolated from bovine articular cartilage was extracted and purified under denaturing conditions as previously described (3). Recombinant biglycan containing a N-terminal polyhistidine tag was produced in HT1080 cells using the vaccinia virus expression system as described (36). The polyhistidine tag was removed and the recombinant proteoglycan was re-isolated as described above. The preparation of recombinant biglycan contain a mixture of proteoglycan and core protein forms (36). The N-terminal segment of the biglycan core protein was produced as a recombinant MBP fusion protein in *E. coli*. The encoded fusion protein MBP-MBGN-N is composed of the maltose binding protein followed by a biglycan peptide, corresponding to amino acid residue 38 to 77. The primers BGNF11 and BGNR13 (Table 1A) and the mouse embryo cDNA library were used to PCR amplify an appropriate cDNA fragment. The resulting PCR product was cleaved, purified, and ligated into the pMAL-p2 vector. Protein expression and purification protocols were the same as those described above in the preparation of MBP-decorin fusion proteins.

Dialysis of the Protein In preparation for Zn^{2+} binding experiments, all proteins were dialyzed against Buffer D (20 mM Tris-HCl, 150 mM NaCl, pH = 7.0) supplemented with 5 mM EDTA (Sigma, St. Louis, MO) to remove possibly contaminating divalent metal ions and subsequently dialyzed against Buffer D (without EDTA) with three changes. Buffer D was shown to contain less than $5x10^{-8}$ M Zn^{2+} ions as analyzed by 4-(2-pyridylazo) resorcinol (PAR) (Sigma, St. Louis, MO) complex formation (40-41).

Zn^{2+}-Chelating Affinity Column Chromatography A resin composed of iminodiacetic acid immobilized Sepharose 6B (Sigma, St. Louis, MO) was charged with ten volumes of 2 mg/ml $ZnCl_2$ (Sigma, St. Louis, MO) in deionized water. The resin was then washed with five volumes

of Buffer E (20 mM Tris-HCl, 150 mM NaCl, pH = 8). The charged Sepharose 6B was packed on top of equal amount of uncharged iminodiacetic acid Sepharose 6B that has been pre-equilibrated in Buffer E. The purpose of having the uncharged resin on the bottom of the column is to trap zinc ions released from the charged resin during the experiment. The dialyzed test sample was applied to the column. The column was washed with excess amount of Buffer E and the bound protein was eluted by Buffer F in which the pH of Buffer E was titered to 4.0 by addition of glacial acetic acid.

Zn^{2+} Equilibrium Dialysis The equilibrium dialysis experiments were carried out in a double acrylic microdialysis module (Hoffer, San Francisco, CA). A dialysis membrane was assembled in between two modules to separate each of the eight chambers into two compartments. The molecular weight cut-off of the dialysis membrane was chosen depending upon the molecular size of the test protein. The 12,000-14,000 Da cut-off membrane (Hoffer, San Francisco, CA) was used for the proteoglycan preparations and the MBP fusion proteins. The 2,000 Da cut-off membrane (Spectra/Pro, Spectrum Medical Industries, Inc, Houston, TX) was used for the MD4 peptide. Aliquots of 150 µl test protein in Buffer D were added to the inner compartments. The same volume of Buffer D containing increasing concentrations of $ZnCl_2$ (0, 2, 4, 8, 16, 24, 32, and 40 µM) was added to the outer compartments. The assembled modules were fitted on a rotating axis and incubated at 4°C for at least 40 h to reach equilibrium.

After incubation, the concentration of Zn^{2+} in the outer compartments was determined using the metallochromic indicator PAR (41). An aliquot of 120 µl from each outer compartment was added to glass tubes containing 2.4 µl of a 5 mM PAR. The components were mixed and incubated for 30 min at room temperature to allow the red PAR-Zn^{2+} complex to form. The absorbance at A_{500} from each sample was determined using a DU-70 spectrophotometer (Beckman, Fullerton, CA). A standard curve of $ZnCl_2$ (0-40 µM) in Buffer D was generated which showed a linear relationship by plotting A_{500} versus the increasing concentration of Zn^{2+}. The concentration of free Zn^{2+} in solution from each outer compartment was determined by inserting the observed absorbance into the standard curve equation.

The concentration of Zn^{2+} bound to protein was determined using equation (A):

$$[Zn^{2+}]_{Total} = 2[Zn^{2+}]_{free} + [Zn^{2+}]_{bound} \quad (A)$$

$[Zn^{2+}]_{Total}$ is the total concentration of Zn^{2+} added to the outside compartment of the equilibrium dialysis chamber at the beginning of the experiment. The $[Zn^{2+}]_{free}$ term is multiplied by a factor of 2 to take into account the dilution of $[Zn^{2+}]_{Total}$ at equilibrium in the absence of protein.

An equation derived for multiple, independent binding sites (equation B) was used to analyze the equilibrium binding data (42):

$$[Zn^{2+}]_{bound}/\text{total protein} = n([Zn^{2+}]_{free}/K_D)/(1 + ([Zn^{2+}]_{free}/K_D)) \quad (B)$$

Total protein is the concentration of protein used in the equilibrium dialysis experiment, n is the number of Zn^{2+} molecules binding to each protein molecule, and K_D is the observed dissociation constant for the Zn^{2+}/protein complex. The reported $K_D \pm$ standard error and Zn^{2+} binding curves represent the average of at least three independent experiments.

RESULTS

Different forms of decorin bind Zn^{2+}.

We recently reported that a Zn^{2+} charged column of iminodiacetate-Sepharose could be used to separate decorin and epiphycan isolated from fetal bovine epiphyseal cartilage (43). In this study, Zn^{2+} chelate affinity chromatography was used to analyze the Zn^{2+} binding activity of different forms of decorin (Fig. 1). Decorin extracted from bovine skin by 4M GdnHCl and purified under denaturing conditions bound to the Zn^{2+} charged column and could be eluted by Tris-acetate buffer pH = 4 (Fig. 1A). Most of the decorin was retained on the affinity matrix but detectable amount of material was recovered in the "pass through" fraction. This "pass through" material migrated as a SLRP when analyzed by SDS-PAGE but it was not further analyzed. Recombinant human decorin produced in HT1080 cells using a vaccinia virus based expression vector and purified under non-denaturing conditions also bound effectively to the Zn^{2+} charged matrix (Fig. 1B). The low molecular weight protein found in "pass through" fractions presumably represent an impurity. This recombinant decorin appears to have a different protein conformation compared to the GdnHCl extracted tissue form which has been denatured (35). Thus, these results suggest that the Zn^{2+} binding property of decorin is not introduced during extraction or a property exclusively found in denatured forms of the proteoglycan.

Equilibrium dialysis was used to characterize the Zn^{2+} -decorin interaction (Fig. 2). The proteoglycan isolated from bovine skin and the recombinant decorin both could be saturated with Zn^{2+} and bound a maximum of two Zn^{2+} ions per decorin molecule with an average K_D of 1.0 ± 0.3 μM and 3.9 ± 1.8 μM, respectively.

The Zn^{2+} binding activity of decorin is located to the core protein.

The recombinant decorin produced in HT1080 cells appears as a mixture of molecular species including proteoglycans and core proteins without glycosaminoglycan chains. Both the

proteoglycan and the core protein forms bound to the Zn^{2+} charged column. The proteoglycans appear as a diffuse band when analyzed by SDS-PAGE, migrating largely between the 68 and 100 K_D molecular standards. The core proteins migrate as a sharper band with an apparent molecular weight of 50 K_D (Fig. 1B). The observation that also the core proteins bound to a Zn^{2+} charged resin suggests that the primary Zn^{2+} binding sites in decorin are located in the core protein. This hypothesis was further examined by analyzing the Zn^{2+} binding properties of prokaryotic recombinants. Recombinant mouse decorin core protein produced in *E. coli* as a fusion protein where the core protein is linked at its N-terminal to the maltose binding protein (MBP) also quantitatively bound to the Zn^{2+} charged matrix (Fig. 1C). MBP alone did not bind to the Zn^{2+} charged matrix (Fig. 1D), neither did isolated dermatan sulfate or chondroitin sulfate polysaccharides (data not shown). Taken together, these results suggest that the core protein alone, is responsible for the Zn^{2+} binding activity of decorin.

The Zn^{2+} binding activity is located to the N-terminal domain of the decorin core protein.

To further locate the domain responsible for the Zn^{2+} binding activity in the core protein, we analyzed recombinant segments of the mouse decorin core protein produced in *E. coli* as fusion proteins linked to the C-terminus of MBP. The different constructs made are shown in Fig. 3. All the fusion proteins were at least partially soluble when produced as described in the Methods Section. Affinity chromatography of bacterial lysate on a column of amylose-Sepharose resulted in a substantial purification of the fusion proteins, although a second purification step was required to obtain reasonably pure proteins. Analyses of the different protein preparations by SDS-PAGE under non-reducing (Fig. 4A) or reducing (Fig. 4B) conditions revealed that when disulfide bonds were disrupted, each preparation contain a major band that migrated as expected based on the deduced molecular weight of the fusion protein. The MBP-MD and MBP-MD1 also contained several minor components of lower molecular weight possibly due to a degradation of the original fusion proteins. In the absence of reducing agent, a substantial portion of MBP-MD, MBP-MD1, MBP-MD3, and MBP-MD4 occurred in

aggregates that did not penetrate the gel. Furthermore, the MBP-MD4 preparations contained two protein forms that penetrated the gel and migrated with apparent molecular weights of 46 kDa (compared to the molecular weight of 50.2 kDa calculated from the sequence of MBP-MD4) and 88 kDa, respectively. The latter possibly representing a dimer. All of these proteins included at least one of the Cys containing end domains of the decorin core protein. The aggregates and dimers were dissociated upon reduction. MBP-MD2 includes only the leucine rich repeat region which does not contain any Cys residues and migrates as a single band to the same position both in the presence and absence of reducing agent.

Chromatography of the different fusion proteins on a Zn^{2+} charged matrix demonstrated that MBP-MD, MBP-MD1, and MBP-MD4 bound Zn^{2+} whereas MBP-MD2, MBP-MD3 did not bind the metal ions (summarized in Fig. 3). The Zn^{2+} binding recombinant proteins all contained the N-terminal segment of the decorin core protein whereas this segment was not present in the non-binders. It is noteworthy that MBP-MD4 which only contains 41 amino acid residues of the N-terminal decorin domain binds efficiently to the Zn^{2+} charged column.

Equilibrium dialyses showed that both MBP-MD and MBP-MD4 could bind Zn^{2+} in a process that exhibits saturation kinetics approaching a maximum of two Zn^{2+} ions per protein (Fig. 5). The average K_D for these interactions was 3.0 ± 1.9 μM and 2.4 ± 0.6 μM, respectively. Binding of Zn^{2+} to MBP-MD2 and MBP-MD3, respectively, could not be demonstrated using equillibrium dialyses (data not shown). These results suggest that the 41 amino acid long N-terminus of decorin retain full zinc binding activity.

Characterization of Zn^{2+} binding to the MD4 peptide.

The experiments described above suggest that the Zn^{2+} binding activity of decorin is located to a relatively short segment from the N-terminal part of the core protein. To further analyze the binding of Zn^{2+} to this segment, we made a new MD4 containing construct using the pGEX-2T vector. The resulting recombinant fusion protein is composed of glutathione S-transferase (GST) followed by a linker and MD4. This fusion protein which is expressed at a

much higher level compared to the MBP based fusion proteins can be purified by affinity chromatography on glutathione-Sepharose. The linker region contains a thrombin cleavage site and preliminary experiments showed that thrombin efficiently cleaved the linker. The released MD4 peptide, which contains four extra amino acid residues (Gly-Ser-Asn-Gly) at the N-terminus followed by residue #31-71 of mouse decorin, was purified by RP-HPLC. The purity of the peptide was verified by SDS-PAGE (Fig. 6). The expected size of the peptide was 4878.56 Da as calculated from the aa sequence and the molecular mass of the isolated peptide was 4878.37 Da as determined by MALDI mass spectroscopy.

The purified MD4 peptide bound to a Zn^{2+} charged iminodiacetate-Sepharose column (data not shown) and equilibrium dialyses experiments showed that the MD4 peptide bound Zn^{2+} ions in a concentration dependent manner that approached a maximum of two Zn^{2+} ions bound per peptide molecule (Fig. 7). Analyses of the binding data assuming the presence of two independent Zn^{2+} binding sites in the MD4 peptide suggest an average K_D of 0.28 ± 0.03 μM for these sites. Analyses of the binding data in a Hill plot did not reveal any pronounced cooperativity between the two binding sites.

The Zn^{2+} binding activity of Biglycan.

The amino acid sequence of the Zn^{2+} binding MD4 decorin peptide is partially conserved in biglycan but less conserved in epiphycan and osteoglycin (Fig. 8). In previous studies, tissue extracted biglycan from bovine articular cartilage have been shown to form multimers in the presence of Zn^{2+} (34). We therefore examined the Zn^{2+} binding activity of different forms of biglycan. Binding to a Zn^{2+} charged column was demonstrated for biglycan isolated under denaturing conditions from bovine articular cartilage or produced as a recombinant proteoglycan in HT1080 cells infected with a recombinant vaccinia virus containing the human biglycan cDNA (data not shown). Equilibrium dialyses showed that both the tissue extracted and recombinant proteoglycan bound a maximum of two Zn^{2+} ions per molecule with an average K_D of 3.3 ± 2.4 μM and 5.4 ± 1.4 μM, respectively (Fig. 9A and B). The N-terminal segment of mouse biglycan

corresponded to amino acid residues #38-77 was expressed in *E. coli* as a recombinant MBP fusion protein (MBP-MBGN-N) and shown to bind to a Zn^{2+} charged column of iminodiacetate-Sepharose (data not shown). Analyses by equilibrium dialyses showed that MBP-MBGN-N could bind a maximum of two molecules of Zn^{2+} with an average K_D of 2.7 ± 1.6 μM (Fig. 9C). These results suggest that biglycan is also a Zn^{2+} binding molecule and that the N-terminus of its core protein has zinc binding activity.

Segments of epiphycan and osteoglycin core proteins containing the N-terminal domains were expressed as fusion proteins (MBP-MEPN-N and MBP-MOGN-N, respectively), purified, and analyzed for Zn^{2+} binding activity. Both fusion proteins bound to the Zn^{2+} charged column (data not shown). In equilibrium dialysis experiments, however, the binding affinity of Zn^{2+} to MBP-MEPN-N and MBP-MOGN-N proteins was too weak ($K_D > 10^{-5}$M) to be detected. These results suggest that residues specifically found in decorin and biglycan but not in epiphycan or osteoglycin are required for forming the high affinity Zn^{2+} binding sites.

DISCUSSION

In this communication, we report that decorin and biglycan are Zn^{2+} metalloproteins. These macromolecules are capable of binding Zn^{2+} regardless of whether they are purified under denaturing conditions from tissues or are produced and isolated under non-denaturing conditions from cultured cells infected by recombinant viruses. Furthermore, the ability to bind Zn^{2+} is not restricted to decorin from one particular specie since we in this study demonstrate a binding of Zn^{2+} to decorin derived from human, mouse or bovine tissue. Decorin and biglycan are members of a growing family of ECM proteins that exhibit high affinity for Zn^{2+}. In addition to matrix metalloproteases, this family includes, laminin (29), link protein (30), nidogen (31) and, COMP (32).

The Zn^{2+} binding activity of decorin is localized to a segment in the N-terminal part of the core protein. A GAG attachment site is also located in this region and involve a Ser residue at position #34 in the mouse decorin sequence. The carbohydrate components of the proteoglycans, however, are not directly involved in Zn^{2+} binding. In fact, a recombinant peptide corresponding to the decorin N-terminal domain and produced in *E. coli* has full Zn^{2+} binding activity. The amino acid sequence of this peptide which contains four Cys residues is reminiscent of a Zn-finger but the spacing of the Cys residues is different from previously reported Zn^{2+} binding motifs (24-25). This apparently novel Zn^{2+} binding sequence must adopt a conformation that allows the binding of two Zn^{2+} ions per peptide. Experiments are currently underway to determine the structure of this peptide in complex with Zn^{2+} ions. Cys residues appear to be involved in coordinating the Zn^{2+} ions since we in preliminary experiments have shown that reduction and alkylation of the MBP-MD4 protein resulted in loss of Zn^{2+} binding activity. Earlier analyses of tryptic peptides obtained from bovine biglycan showed that the first and the forth Cys residues in the N-terminal domain are linked through a disulfide bond (44). This observation leaves the second and third cys residues as potential Zn^{2+} coordinators. If in fact the Cys residues are involved in Zn^{2+} binding, this could help explain the different affinities

we see for Zn^{2+} among the different forms of decorin tested. The isolated MD4 peptide which was isolated as a homogenous form by RP-HPLC has the highest apparent affinity for Zn^{2+}.

The Zn^{2+} binding MBP fusion proteins all occurred as mixtures of several molecular forms where some Cys residues appear to be engaged in forming disulfied linked multimers. The measured K_D's for Zn^{2+} binding to these proteins were higher compared to that recorded for the isolated MD4 peptide. Perhaps Cys residues engaged in coordinating Zn^{2+} in the MD4 peptides are in the multimers involved in disulfide linkages. Alternatively, this observation may suggest that structures outside the actual binding site may influence the Zn^{2+} binding activity of this domain. Thus the isolated sequence present in the peptide appears to have a higher affinity for Zn^{2+} than when the sequence is part of a larger structure as in an intact core protein or a proteoglycan. Decorin contains in addition to a traditional N-terminal signal peptide that is removed on secretion, a 13 amino acid long propeptide, which also often has been removed from the proteoglycan isolated from tissues. The decorin MD4 peptide or the proteoglycan forms here shown to bind Zn^{2+} with a high affinity do not contain the propeptide. It is unclear if the presence of a propeptide may affect the Zn^{2+} binding to decorin or biglycan.

Recombinant N-terminal segments of epiphycan and osteoglycin core protein made as MBP fusions, which contain sequences similar to the Zn^{2+} binding sequences present in decorin and biglycan, also are retained on a Zn^{2+} charged iminodiacetate-Sepharose in this study. However, the affinity of the MBP-fusion proteins for Zn^{2+} is too low to measure reliable binding constants by equilibrium dialyses. In an earlier study, we found that Zn^{2+} chelating chromatography could be used to fractionate tissue extracted epiphycan (which did not bind to the column) from decorin (which bound to the column). This result is in contrast to the chromatography data obtained in the current study and could be explained if the full length epiphycan proteoglycan has a lower affinity for Zn^{2+} compared to the recombinant N-terminal peptide. Such a pattern was established for decorin. In the N-terminal segments of epiphycan and osteoglycin, the Cys residues are spaced slightly differently than in the Zn^{2+} binding peptides of decorin and biglycan; there are two compared to three amino acid residues between

the first and second Cys in epiphycan, osteoglycin and decorin/biglycan, respectively. It is possible but not yet demonstrated that the spacing of Cys residues determines the Zn^{2+} binding activity of these segments.

The concentration of Zn^{2+} in body fluids such as blood plasma is approximately 15μM, which suggests that both decorin and biglycan *in vivo* occur in complex with Zn^{2+}. The significance of this complex is unclear. Decorin and biglycan which are abundant molecules in the tissues could serve as Zn^{2+} storage pools where the metal ions could be released to proteins which that have a higher affinity for and need Zn^{2+} ions for their activity. In addition, Zn^{2+} may stabilize a conformation in the proteoglycan core proteins which are important for their functions. In fact preliminary results obtained by analyses of the MD4 peptides using circular dichroism spectroscopy suggest that the conformation of the peptide is altered in the presence of Zn^{2+}. This observation is in agreement with that of Liu et al. (34) who found that biglycan had a tendency to aggregate in the presence of Zn^{2+} which could be caused by a conformational change in the protein induced by Zn^{2+} binding. Future studies of the biology of decorin and biglycan must take into account the fact that these molecules are Zn^{2+} metalloproteins.

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ABBREVIATIONS

[¶]The abbreviations used are: ECM, extracellular matrix; SLRP, small leucine rich proteoglycan; PRELP, proline-arginin-rich and leucine-rich repeat protein; TGFß, transform growth factor ß; COMP, cartilage oligomeric matrix protein; MBP, maltose binding protein; IPTG, isopropylthio-ß-galactosidase; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; MALDI-MS, matrix assisted laser desorption ionization-mass spectroscopy; EDTA, ethylenediamine tetraacetic acid; PAR, 4-(2-pyridylazo) resorcinol; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

FIGURE LEGENDS

Figure 1 **Binding of Zn^{2+} to different forms of decorin by Zn^{2+}-chelating affinity column chromatography.** Bovine skin decorin proteoglycan (BDCN) (A), recombinant human decorin proteoglycan (HDCN) (B), recombinant maltose binding protein -mouse decorin core protein fusion (MBP-MD) (C), and maltose binding protein (MBP) (D) were analyzed for Zn^{2+} binding activity. 0.5 mg protein was applied to 1 ml Zn^{2+}-charged column. The column was extensively washed and the protein was subsequently eluted by Tris-acetate buffer at pH = 4. Fractions of 0.5 ml were collected and monitored by A_{280}. The arrow indicates where the elution buffer was applied. The samples were also analyzed by SDS-PAGE on 7.5% [(A) and (B)] and 10% [(C) and (D)] polyacrylamide gels under reducing conditions. (S) represents 30 µl of the starting material; (P) represents fractions 1-5 which were combined, concentrated to dryness, and resuspend in 30 µl; (E) represents fractions 12-16 which were combined, concentrated to dryness, and resuspend in 30 µl. The gels were stained with Coomassie Brilliant Blue R-250.

Figure 2 **Zn^{2+} binding to decorin; equilibrium dialysis.** Decorin extracted from fetal bovine skin (BDCN) or produced as a recombinant using a vaccinia virus vector (vvHDCN) were examined for their Zn^{2+} binding activity using equilibrium dialysis. For further details see the "Experimental Procedure" section. The K_D and number of binding sites (n) ± standard error represents the average of three experiments. The correlation coefficients for the binding curves were 0.95.

Figure 3 **Constructs of decorin core protein fragments.** The diagram shows the recombinant decorin constructs expressed in *E. coli*, their molecular weight (Mw), and summarizes the results of the different fusion protein's ability to bind to a Zn^{2+} charged iminodiacetic acid column.

Figure 4 **Analyses of purified recombinant MBP-decorin fusion proteins by SDS-PAGE.** The purified MBP-decorin fusion proteins were loaded in non-reducing sample buffer (A) and reducing buffer containing 10mM DTT (B), respectively, on 12% polyacrylamid gels. The gels were stained with Coomassie Brilliant Blue R-250. The migration of standard proteins of the indicated Mw is shown in the left panel.

Figure 5 **Analyses of Zn^{2+}-binding to MBP-MD and MBP-MD4 by equilibrium dialysis.** The Zn^{2+} binding of MBP-MD and MBP-MD4 was analyzed by equilibrium dialysis as outlined in the "Experimental Procedure" section. The data shown represents an average of triplicate experiments. The correlation coefficients of the binding curves were 0.9.

Figure 6 **Isolation and purification of decorin N-terminal peptide.** The decorin N-terminal peptide MD4 was expressed as a GST-MD4 fusion protein, cleaved by thrombin digestion and isolated by RP-HPLC, as described in the "Experimental Procedure" section. The purity of the peptide was analyzed by tricine buffered SDS-PAGE. Lane 1, glutathione agarose purified GST-MD4 fusion protein, lane 2, RP-HPLC purified GST carrier after thrombin cleavage, and lane 3, RP-HPLC purified MD4 peptide. The migration of standard proteins of the indicated Mw is shown in the left panel. The 8.2 and 6.2 kDa protein markers are the products of CNBr cleaved fragments from horse heart myoglobin.

Figure 7 **Zn^{2+} binding affinity of MD4 peptide.** Equilibrium dialysis was used to analyze the binding of the isolated MD4 peptide to Zn^{2+} as described in the "Experimental Procedures" section. Each data point was the average of triplicate experiments. The correlation coefficient was 0.99.

Figure 8 **Partial N-terminal amino acid sequence of mouse decorin, biglycan, epiphycan, and osteoglycin.** The 41 amino acid sequence of N-terminus of mouse decorin (MDCN) which has Zn^{2+} binding activity is shown and compared to the corresponding sequences of mouse biglycan (MBGN), epiphycan (MEPN), and osteoglycin (MOGN). One amino acid space was added in MEPN and MOGN to obtain an alignment of the four cys residues in these sequences. The numbering of encoding amino acid sequence was based on the protein sequence deposited in GenBank.

Figure 9 **Zn^{2+} binding affinity of different forms of biglycan.** The Zn^{2+} binding of biglycan extracted from bovine articular cartilage (BBGN), produced in culture mammalian cells using a vaccinia virus vector (vvHBGN), or a N-terminal peptide expressed as a maltose binding protein fusion (MBP-MBGN-N) were analyzed by equilibrium dialysis as described in the "Experimental Procedures" section. Each binding curve was plotted from the average of triplicate experiments. The correlation coefficients were 0.93.

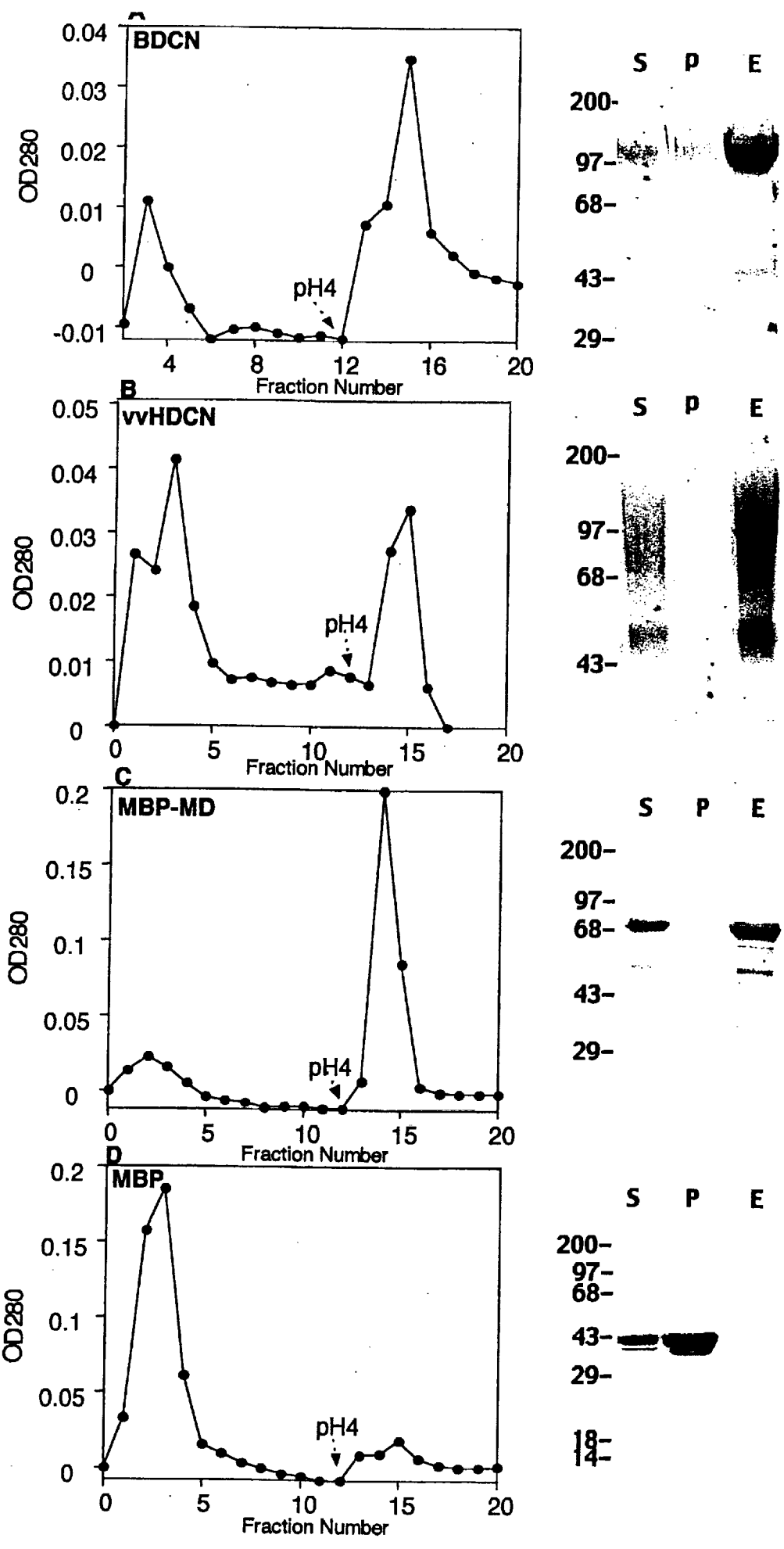
A. Primers for PCR amplyfication of different decorin and biglycan fragments

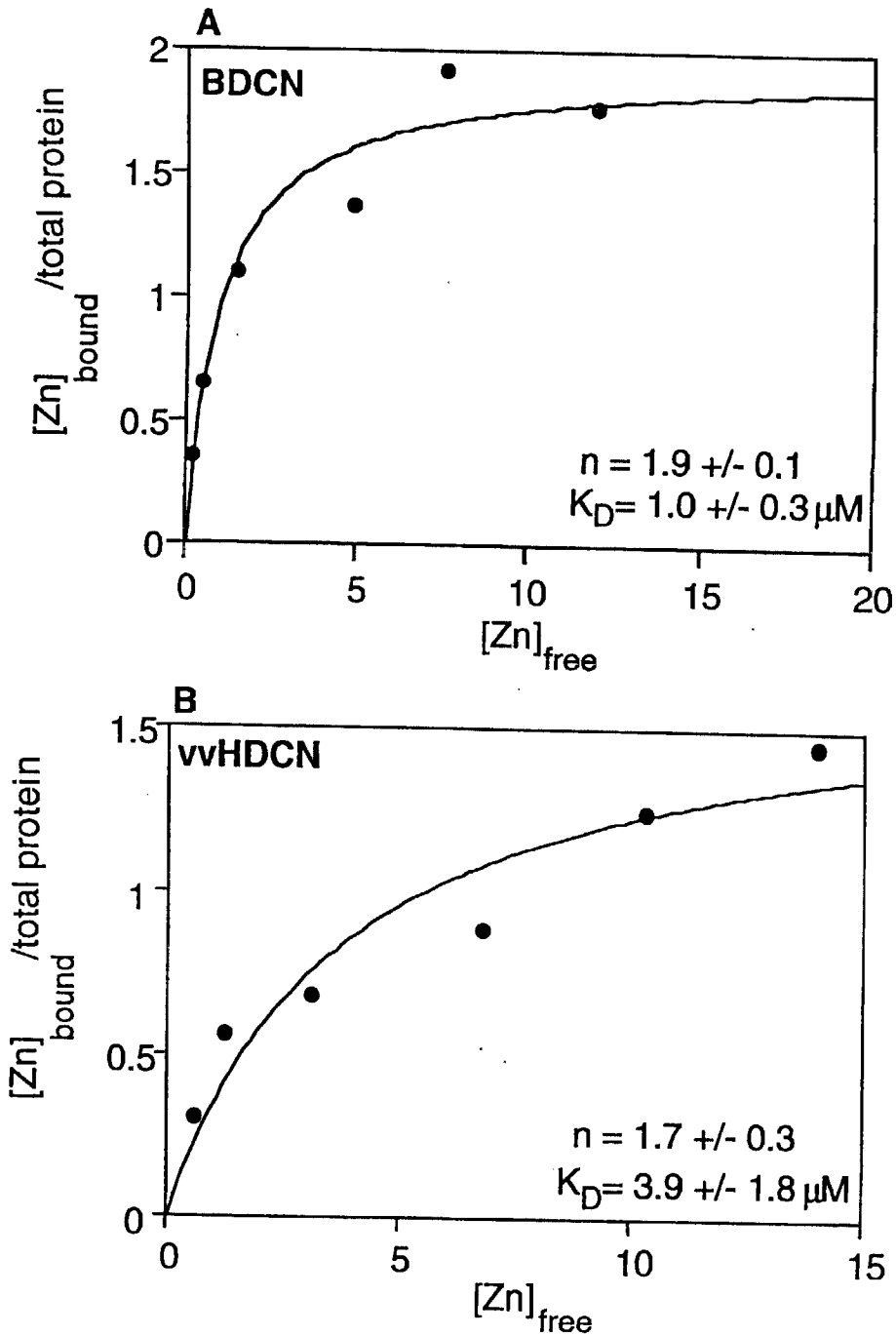
DCNF9	cgcggatccgatgaggcttctggcataatc	
	BamHI D E A S G I I	
DCNR2	ccggaattcttacttgtagtttccaagttgaatggc	
	EcoRI * K Y N G L Q I A	
DCNR18	ccccctgcagttacccaactgcggagatgttgtt	
	Pst I * G V A S I N N	
DCNF17	gccggatcctgggattttccacccgacac	
	BamHI W D F P P D	
DCNR19	ccccctgcagttagggcactttgtccaa	
	Pst I * P V K D L	
BGNF11	ggcggatccgatgaggaggcttcaggttca	
	BamHI D E E A S G S	
BGNR13	cccgaattcctagcactgaacaacccgcag	* stop codon
	EcoRI * C Q V V R L	

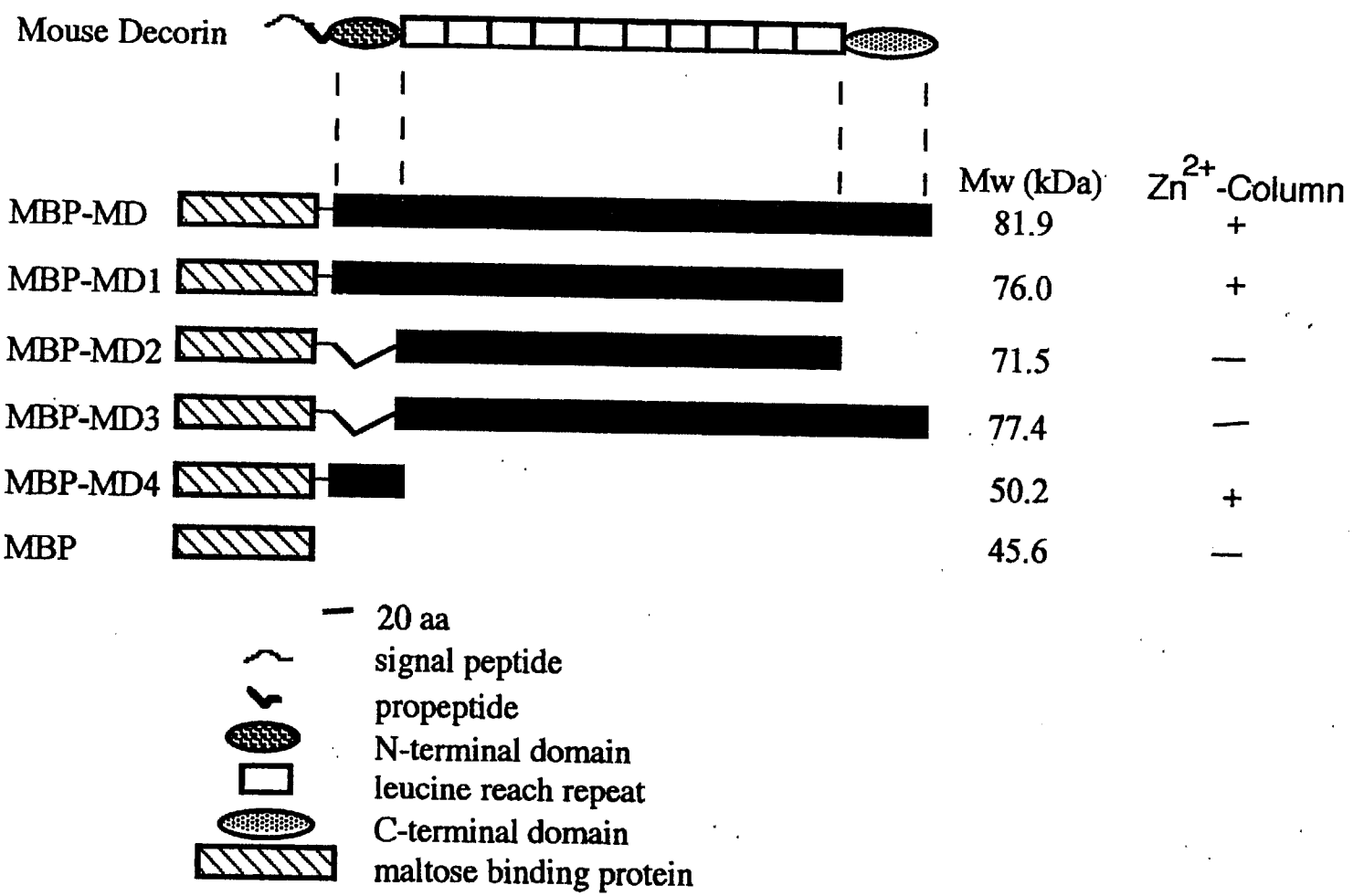
B. The corresponding primer pairs for expressed protein subdomains

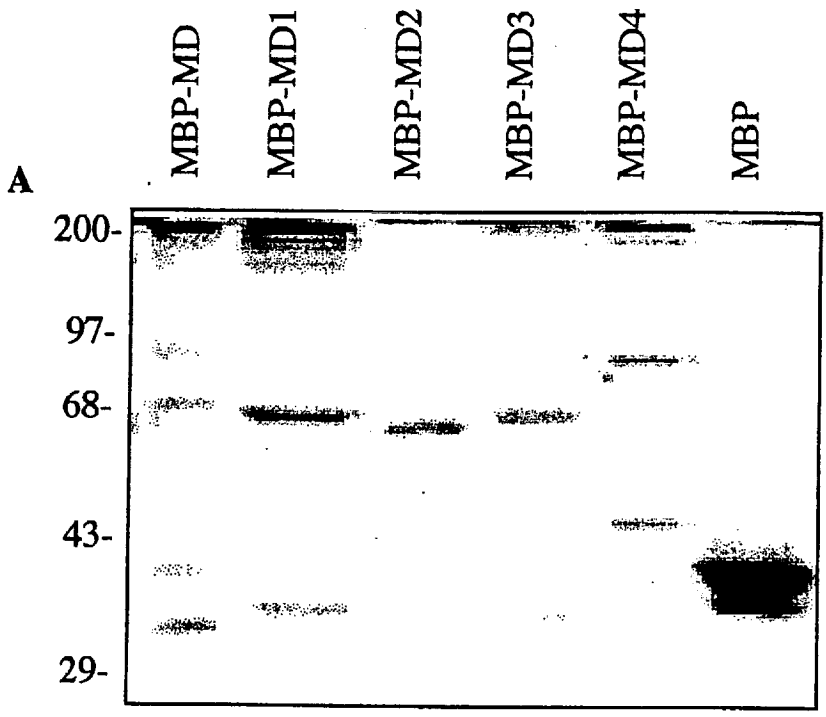
Fragment	Primers	Number of base pairs[a]	Number of amino acid residue[b]
MD	DCNF9 DCNR2	232 - 1296	31 - 354
MD1	DCNF9 DCNR18	232 - 1140	31 - 303
MD2	DCNF17 DCNR18	445 - 1140	72 - 303
MD3	DCNF17 DCNR2	445 - 1296	72 - 354
MD4	DCNF9 DCNR19	232 - 444	31 - 71
MBGN-N	BGNF11 BGNR13	346-465	38 - 77

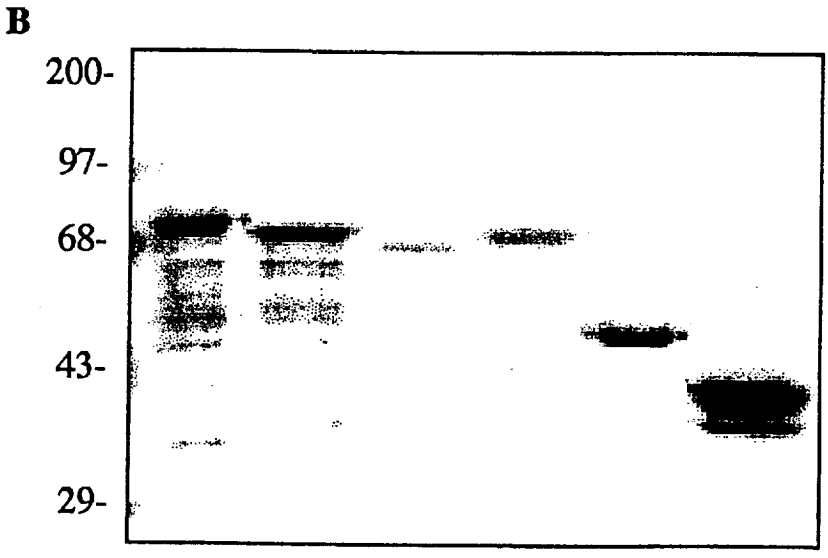
[a,b]The nucleotide assigned number and amino acid number is based on the mouse decorin (PGS2) sequence search from data base, accession number P28654 and mouse bigycan (PGI), accession number X53928.

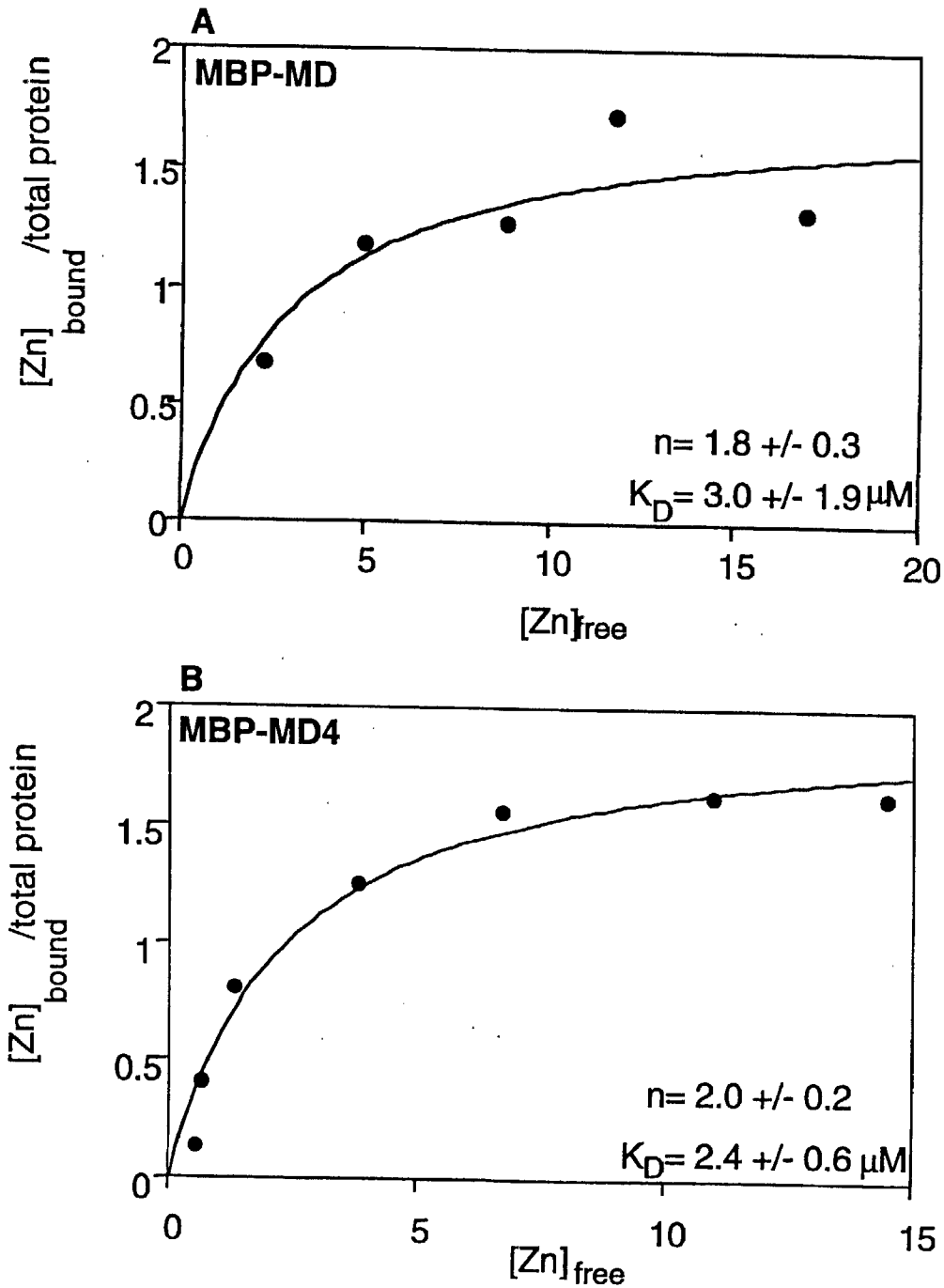

A
BDCN
OD280
0.04
0.03
0.02
0.01
0
-0.01
pH4
4
8
12
16
20
Fraction Number
S P E
200-
97-
68-
43-
29-
B
vvHDCN
OD280
0.05
0.04
0.03
0.02
0.01
0
pH4
0
5
10
15
20
Fraction Number
S P E
200-
97-
68-
43-
C
MBP-MD
OD280
0.2
0.15
0.1
0.05
0
pH4
0
5
10
15
20
Fraction Number
S P E
200-
97-
68-
43-
29-
D
MBP
OD280
0.2
0.15
0.1
0.05
0
pH4
0
5
10
15
20
Fraction Number
S P E
200-
97-
68-
43-
29-
18-
14-

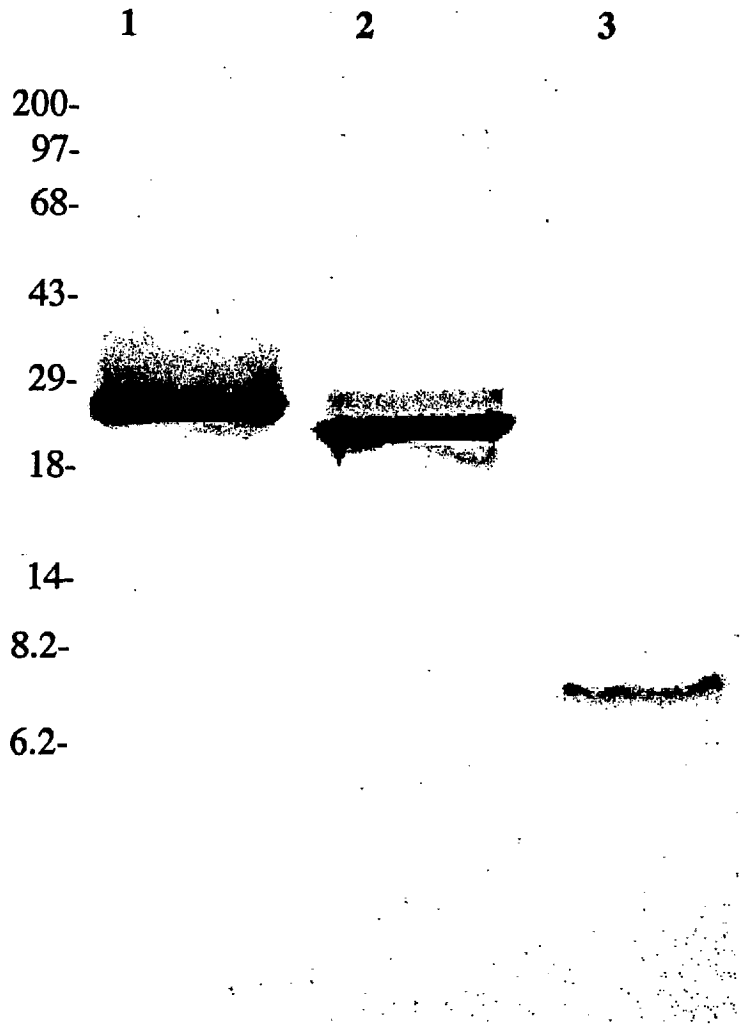

A
BDCN
[Zn]bound /total protein
2
1.5
1
0.5
0
0
5
10
15
20
[Zn]free
n = 1.9 +/- 0.1
KD= 1.0 +/- 0.3 µM
B
vvHDCN
[Zn]bound /total protein
1.5
1
0.5
0
0
5
10
15
[Zn]free
n = 1.7 +/- 0.3
KD= 3.9 +/- 1.8 µM

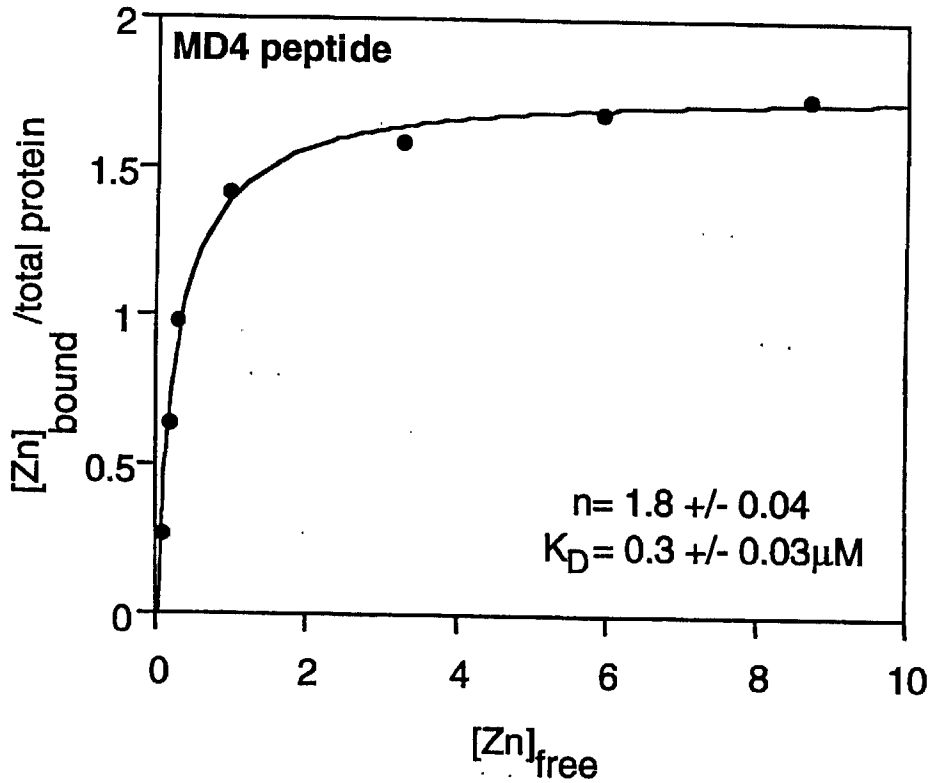

Mouse Decorin
MBP-MD
MBP-MD1
MBP-MD2
MBP-MD3
MBP-MD4
MBP
Mw (kDa)
81.9
76.0
71.5
77.4
50.2
45.6
Zn2+-Column
+
+
—
—
+
—
20 aa
signal peptide
propeptide
N-terminal domain
leucine reach repeat
C-terminal domain
maltose binding protein

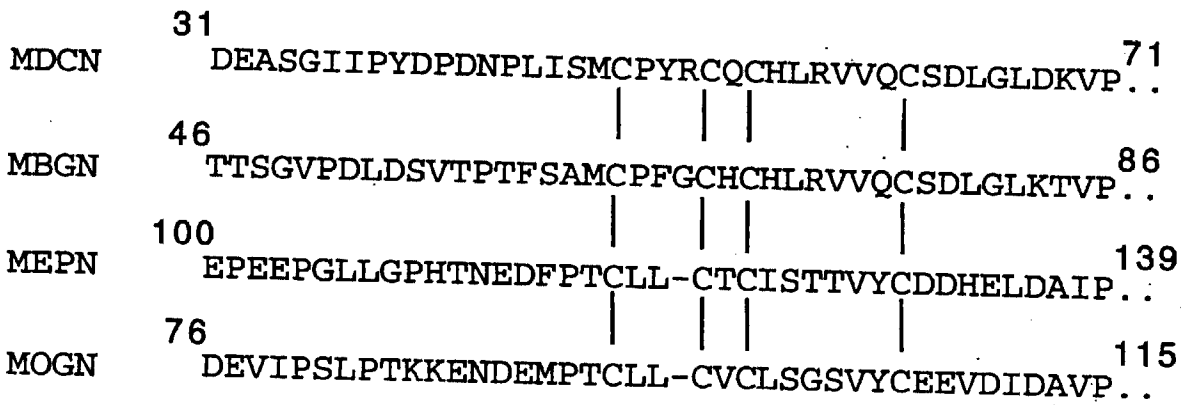

A
MBP-MD
MBP-MD1
MBP-MD2
MBP-MD3
MBP-MD4
MBP
200-
97-
68-
43-
29-


B
200-
97-
68-
43-
29-


A
MBP-MD
[Zn] bound /total protein
[Zn]free
n= 1.8 +/- 0.3
K_D= 3.0 +/- 1.9 µM
B
MBP-MD4
[Zn] bound /total protein
[Zn]free
n= 2.0 +/- 0.2
K_D= 2.4 +/- 0.6 µM


1
2
3
200-
97-
68-
43-
29-
18-
14-
8.2-
6.2-


MD4 peptide
[Zn]bound /total protein
[Zn]free
n= 1.8 +/- 0.04
KD= 0.3 +/- 0.03µM
0
0.5
1
1.5
2
0
2
4
6
8
10


31
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46
MBGN TTSGVPDLDSVTPTFSAMCPFGCHCHLRVVQCSDLGLKTVP 86
100
MEPN EPEEPGLLGPHTNEDFPTCLL-CTCISTTVYCDDHELDAIP 139
76
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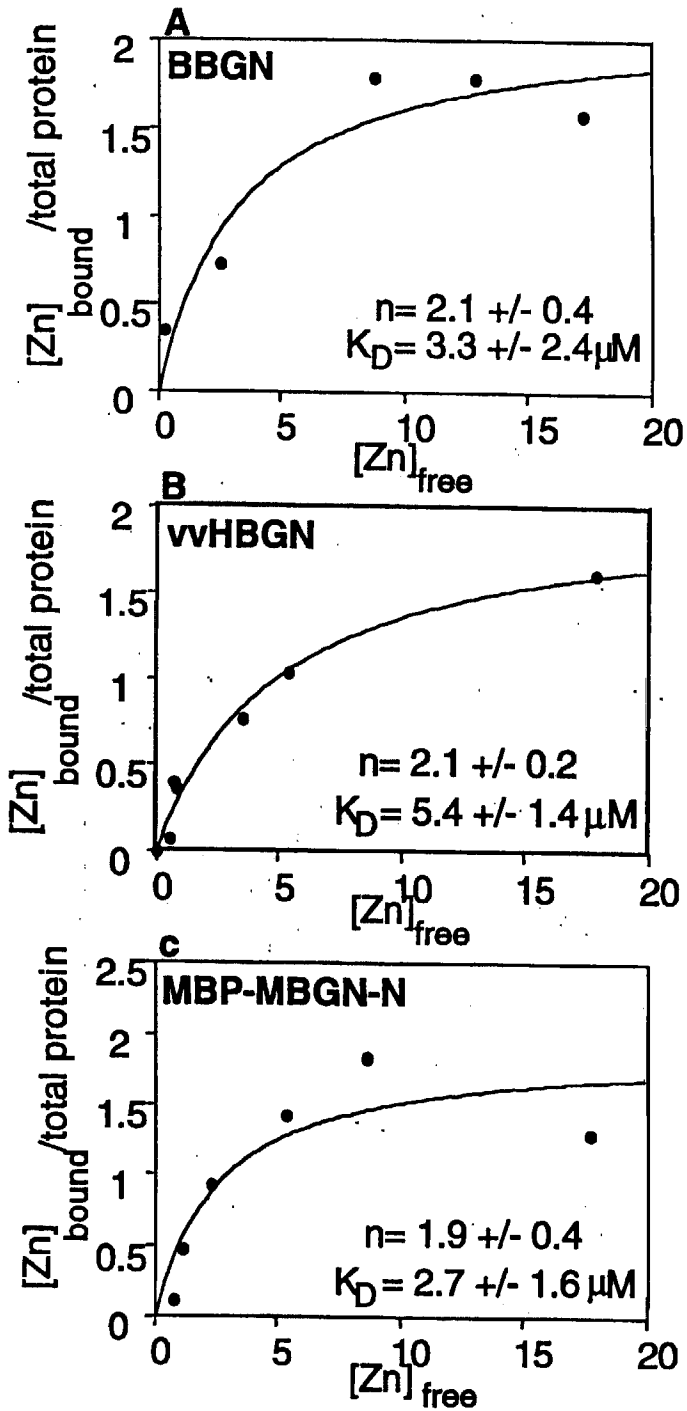
A
BBGN
n= 2.1 +/- 0.4
K_D= 3.3 +/- 2.4µM
[Zn]bound /total protein
[Zn]free
B
vvHBGN
n= 2.1 +/- 0.2
K_D= 5.4 +/- 1.4 µM
[Zn]bound /total protein
[Zn]free
c
MBP-MBGN-N
n= 1.9 +/- 0.4
K_D= 2.7 +/- 1.6 µM
[Zn]bound /total protein
[Zn]free