Characterization of Recombinant Amino-terminal NC4 Domain of Human Collagen IX

INTERACTION WITH GLYCOSAMINOGLYCANS AND CARTILAGE OLIGOMERIC MATRIX PROTEIN*

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The N-terminal NC4 domain of collagen IX is a globular structure projecting away from the surface of the cartilage collagen fibril. Several interactions have been suggested for this domain, reflecting its location and its characteristic high isoelectric point. In an attempt to characterize the NC4 domain in more detail, we set up a prokaryotic expression system to produce the domain. The purified 27.5-kDa product was analyzed for its glycosaminoglycan-binding potential by surface plasmon resonance and solid-state assays. The results show that the NC4 domain of collagen IX specifically binds heparin with a K_d of 0.6 μM, and the full-length recombinant collagen IX has an even stronger interaction with heparin, with an apparent K_d of 3.6 μM. The heparin-binding site of the NC4 domain was located in the extreme N terminus, containing a heparin-binding consensus sequence, whereas electron microscopy suggested the presence of at least three additional heparin-binding sites on full-length collagen IX. The NC4 domain was also shown to bind cartilage oligomeric matrix protein. This interaction and the association of cartilage oligomeric matrix protein with other regions of collagen IX were found to be heparin-competitive. Circular dichroism analyses of the NC4 domain indicated the presence of stabilizing disulfide bonds and a thermal denaturation point of about 80 °C. The pattern of disulfide bond formation within the NC4 domain was identified by tryptic peptide mass mapping of the NC4 in native and reduced states. A similar pattern was demonstrated for the NC4 domain of full-length recombinant collagen IX.

Collagen IX is a heterotrimer of α1(IX), α2(IX), and α3(IX) polypeptide chains that fold into the triple helix characteristic of the members of the collagen family of extracellular matrix proteins (1). This helix consists in the case of collagen IX of COL1, COL2, and COL3 domains, numbered from the carboxyl terminus, which is flanked by short noncollagenous segments, domains NC1–NC4. The domain NC4 is formed by the 245 extreme N-terminal amino acid residues of the α1(IX) chain, since a corresponding region is absent from the α2(IX) and α3(IX) polypeptides (2).

The function of collagen IX remains elusive. It is a minor component of the collagen fibrils of cartilage extracellular matrix and is also found in several other tissues. Collagen IX molecules are not present within the fibril body in cartilage but are instead associated with the surface of the collagen fibril and become covalently cross-linked to other collagen IX molecules and to collagen II, the major constituent of the fibril (2, 3). Collagen IX is not required for the assembly of the heterotypic collagen fibrils, but it is important for preservation of the long term stability of the cartilage extracellular matrix (4, 5). The molecular mechanism involved is not understood, however. The NC4 domain of collagen IX is seen in electron microscopy as a compact globulus projecting away from the fibril body, with the COL3 domain acting as a spacer arm (6, 7). This location and the high theoretical pl of the NC4 domain implicate collagen IX as a potential docking molecule, possibly connecting the host fibril to adjacent collagen fibrils or to other macromolecules of the extracellular matrix (8). Proteoglycans of the cartilage extracellular matrix may serve an intermediary purpose in these processes. A proteolytic fragment of collagen IX, lacking the NC4 domain and some other parts of the molecule, is indeed known to bind heparin with high affinity in vitro (9). The NC4 domain reportedly shows homology to the heparin-binding N-terminal domain of thrombospondin, but the residues believed to be crucial for heparin-binding potential of thrombospondin are not conserved in the NC4 domain (10). No research has yet been reported, however, on the glycosaminoglycan binding properties of the NC4 domain or full-length collagen IX.

Studies in vitro have demonstrated that cartilage oligomeric
matrix protein (COMP) is able to bind collagen IX and collagen II with high affinity in a $\text{Zn}^{2+}$-dependent fashion (11, 12). The collagen IX molecule appears to possess four binding sites for the C-terminal globular domain of COMP, one at or near each NC domain of the protein (11, 12). According to a recent report, another oligomeric protein of the cartilage matrix, matrilin-3, also binds to collagen IX and collagen II in a $\text{Zn}^{2+}$-dependent fashion (13) and has been shown to interact with COMP in a manner that is inhibited by cations $\text{Zn}^{2+}$ and $\text{Ca}^{2+}$ (13). Although direct evidence is lacking, it seems possible that these in vitro interactions reflect the potential of collagen IX, COMP, and matrilin-3 for forming a macromolecular complex in a controlled fashion in vivo. Interestingly, mutations in these three proteins are known to cause a similar phenotype, multiple epiphyseal dysplasia, MED (13). This form of osteochondrodysplasia is a diverse disease both genetically and phenotypically and is typically manifested in irregular epiphyses of the long bones and in most cases early onset osteoarthritis. All mutations thus far characterized in the three genes encoding collagen IX result in an in-frame deletion of at least 12 amino acids from the COL3 domain of the respective component polypeptide. Despite this intriguing finding and the existence of detailed information on mutations found in the genes encoding COMP and matrilin-3, the molecular mechanism of the pathogenesis of MED is not yet properly understood (13).

In the present work, we describe the production and characterization of the NC4 domain of human collagen IX as a recombinant protein and report on the interaction properties of this domain and its parental molecule with heparin and COMP.

**EXPERIMENTAL PROCEDURES**

**Generation of the Bacterial Strain Expressing the Collagen α1(IX) NC4 Domain**—To generate a DNA construct for expression of the NC4 domain of the human α1(IX) polypeptide, oligonucleotide primers were designed to amplify the region corresponding to amino acids 24–268 of the full-length polypeptide (NCB accession number NP_001842), thereby omitting the 23-amino acid signal peptide (14). The 5'-primer (GGA TCC ATG GTU ATC GAA GGT CGA GCT GTC AAG CGT CGC) contained an engineered NcoI cleavage site, and the 3'-primer (GAC TGA ATT CTG ATC TCT GTG CGG TGG TCT G) contained an engineered NdeI cleavage site. Since the respective domain of the avian α1(IX) chain had previously been produced as a C-terminal GST fusion protein (15), a similar expression strategy was chosen here. Consequently, the 5'-primer contained an additional sequence to generate a cleavage site for Factor Xa protease between the fusion partner and the NC4 domain. A previously characterized viral expression construct for the full-length collagen α1(IX) chain (14) was used as a template in PCR. The amplification product was digested with the enzymes indicated above and ligated into the pGAT-2 bacterial expression vector, resulting in pNC1 (16). This vector contained the sequences for the GST fusion tag and had been constructed to be compatible with the pGAT-2 bacterial expression vector. pGAT-2-NC1 with a mutation converting the Cys219 to Ser was ligated into the pGAT-2 bacterial expression vector in frameset with the sequences for the GST fusion tag. Following transformation, the recombinant NC4 domain (rNC4) was cleaved off from the fusion partner and its parental molecule with heparin and COMP.

**Expression and Purification of Recombinant Collagen IX and COMP**—Full-length collagen IX was expressed in shaker flasks by inoculating an aliquot of the frozen E. coli BL21(DE3) cell line directly into the desired final volume of LB broth supplemented with 0.1 mM IPTG and 0.1 mM DTT. The induction was continued at 37 °C for 4–6 h. Following centrifugation, the cell pellets were stored frozen and later homogenized on ice by sonication in 0.3 M NaCl, 0.25% Igepal CA-630 (Sigma), and 0.05 M sodium phosphate buffer, pH 7, supplemented with 0.25 mg/ml lysozyme. Insoluble material was removed by centrifugation at 17,000 × g for 40 min at 4 °C, and the fusion protein was precipitated from the supernatant by adding ammonium sulfate to 30% saturation. The precipitate was collected by centrifugation at 23,000 × g for 30 min at 4 °C and dissolved in PBS supplemented with 1% Igepal CA-630. The solution was clarified by centrifugation and incubated with a 1:20 volume of a 50% slurry of glutathione-Sepharose (Amersham Biosciences) in PBS at 4 °C for 1 h. Following removal of the unbound material by centrifugation and three washing steps with PBS, the recombinant NC4 domain (rNC4) was cleaved off from the fusion partner and its parental molecule with heparin and COMP. The size and purity of the product were determined by both SDS-PAGE analysis and electrospray ionization mass spectrometry. The identity of the purified protein was verified by amino acid analysis in an Applied Biosystems 421 analyzer and by analysis of the tryptic peptides by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The concentration of purified rNC4 was determined in a dye-binding assay (Bio-Rad) calibrated by quantitative amino acid analysis of rNC4.

**Circular Dichroism Analyses**—CD spectra were measured using a Jasco J-810 instrument. Far-UV spectra were measured using a 0.1-cm path length cell for the 185–250-nm region, with a step size of 0.1 nm, scan speed of 20 mm/min, response of 0.25 s, bandwidth of 1 nm with a 250-nm region, with a step size of 5° C in steps of 5°C and at a rate of 1°C/min, allowing stabilization for 2 min before scanning. Lyophilized rNC4 was dissolved in water at a concentration of 10 μM. Measurements under reduced conditions were performed in water supplemented with 1 μM DTT. The temperature scan experiments were performed at the wavelength of 205 nm, raising the temperature from 10 to 90 °C at a rate of 0.5°C/min. Near-UV CD spectra were measured for 105 μM NC4 in 1 mM HEPES buffer, pH 7.4, in the presence or absence of a 10-fold molar excess of 17–19-kDa heparin (Sigma), using a 0.5-cm path length cell for the 250–320-nm region. Other parameters were as above.

**Preparation of Biotinylated Small Unilamellar Vesicles**—Biotinylated small unilamellar vesicles (SUV) for the surface plasmon resonance studies were prepared by a method adapted from those described earlier (17, 18). A solution of egg yolk 1,2-phosphatidylcholine (Sigma) in chloroform/methanol (9:1) containing 2% biotinylated phosphatidylcholine (biotin DHPE, Molecular Probes Europe BV) was dried under a stream of nitrogen at 37 °C, water bath and lyophilized in a vacuum for 2 h. Multilamellar vesicles were obtained by quickly hydrating the dried mixture with HBS buffer (150 mM NaCl and 20 mM HEPES buffer, pH 7.4). The solution was subjected to probe sonication until the sample became clear (5 × 3 min on a cool water bath using a microtip probe), and the resulting SUV were purified by ultracentrifugation at 100,000 g for 30 min. The top third of the supernatant was removed and stored, protected from light, at 4 °C for use in the surface plasmon resonance studies.

**Surface Plasmon Resonance Assay**—The surface plasmon resonance assays were performed on a Biacore 2000™ instrument using either streptavidin-coated SA or hydrophobic HPA sensor chips (Biacore AB), and kinetic parameters were determined with the manufacturer's Biacore evaluation 3.02 software version. The flow cell was held at 22°C and $k_{on}$ and $k_{off}$ rate constant measurements according to the Langmuir binding model. The hydrophobic surface of the HPA sensor chips was coated with biotinylated SUV as suggested by the manufacturer. The biotin-binding protein NeutrAvidin™ (Pierce), dissolved in HBS, was injected over the biotinylated lipid surface to obtain an active surface for binding biotinylated ligands.

To study the interactions of the analytes with heparin surfaces, high molecular mass (17–19 kDa) heparin (Sigma) was biotinylated by incubation with an excess of N-hydroxysuccinimidobiotin (Pierce), followed by dialysis in water and lyophilization, and dissolved in HBS buffer.
The biotinylated heparin was injected over the NeutrAvidin™ or streptavidin surface in a Biacore instrument, and the remaining binding sites were blocked by injecting dilute uncoupled biotin. One of the four flow paths in each sensor chip was left free of bound heparin to provide a negative control. Either rNC4 or recombinant human collagen IX (rIX) produced in insect cells (14) was injected as the analyte at various concentrations at a flow rate of 10 or 25 μl/min for 2 or 5 min in HBS buffer. To study the specificity of interaction with heparin, a constant amount of analyte was preincubated with different amounts of heparin, chondroitin 6-sulfate, or dermatan sulfate (Sigma) prior to injection. The surfaces were regenerated after each injection cycle with repeated injections of a solution containing 0.61 mM NaCl and 15 mM HEPES, pH 7.4.

To study the interaction between NC4 and COMP, an aliquot of rNC4 was biotinylated at its carboxyl groups by incubation with a 10-fold molar excess of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride solution and a 100-fold molar excess of biotin-PDE-amine (Pierce), followed by dia lysis and lyophilization, and dissolved in HBS buffer containing 0.5 mM ZnCl₂. Biotinylated rNC4 was attached to a biacore sensor chip in a similar fashion as above, but supplementing the HBS buffer with 0.5 mM ZnCl₂ (19), and the interaction between COMP and rNC4 was studied by injecting various amounts of COMP (11) at a flow rate of 10 μl/min with a 10-min contact time. The surface was regenerated after each cycle with three injections of a solution containing 0.61 mM NaCl, 15 mM HEPES, and 10 mM EDTA, pH 7.4. The kinetics of the interaction between NC4 and COMP were determined by using COMP T3 + TC (16), which was biotinylated by incubation with a 2-fold molar excess of biotinamidocaproate N-hydroxysuccinimide ester (Sigma) in 0.2 mM NaHCO₃, pH 8.2, followed by dialysis and lyophilization, and dissolved in HBS buffer containing 0.5 mM ZnCl₂. Biotinylated COMP T3 + TC was attached to the surface of a sensor chip as above, and rNC4 or rIX was injected over the surface in the presence or absence of excess heparin at a flow rate of 40 μl/min with a 2 min contact time. After each cycle, the surface was regenerated with two injections of 10 mM EDTA in HBS.

Solid-phase Heparin-binding Assays—For solid-state binding assays, rNC4 or rIX at 1 μg/ml in PBS (0.137 M NaCl, 10 mM phosphate buffer, pH 7.4) or KCl were coated on streptavidin or NeutrAvidin™ (Nunc, Denmark) overnight at 4 °C. Further binding was performed with 1/50 bovine serum albumin in PBS. Heparin–albumin–biotin (Sigma) at 5 μg/ml or biotinylated heparin (see above) at 36 μg/ml in PBS supplemented with 0.1% bovine serum albumin and 0.05% Tween 20 was allowed to interact with rNC4 or collagen IX surface, respectively, in the absence or presence of a nonbiotinylated competitor. The levels of bound biotinylated reagents were detected with horse radish peroxidase-conjugated NeutrAvidin™ using o-phenylenediamine dihydrochloride (Pierce) as substrate. The low background levels from wells coated with bovine serum albumin were subtracted from the experimental data.

Electron Microscopy—Bovine serum albumin was coupled to high molecular weight heparin by incubating with a 20-fold molar excess of heparin and a 200-fold molar excess of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride solution for 2 h at room temperature in 0.1 M MES buffer, pH 5.0. The obtained heparin–bovine serum albumin was purified by gel filtration chromatography on Superdex 75 (Amersham Biosciences), dialyzed, conjugated with colloidal gold of 4 nm, and allowed to react with full-length collagen IX. Formed complexes were visualized by electron microscopy after negative staining with uranyl formate. Preparation of gold conjugates and electron microscopy were performed as described recently in detail (20).

Identification of the Heparin-binding Region of the NC4 Domain—The NC4 domain was reduced with DTT and alkylated with iodoacetamide. Free iodoacetamide was reduced with 0.1 M DTT and removed by dialysis into 50 mM ammonium acetate buffer, pH 4.0. A 450-μg aliquot of the alkylated rNC4 was subjected to digestion with 12 units of V8 protease (Sigma) in the same buffer at room temperature for 4 h. The reaction mixture was diluted 4-fold with 0.1 M ammonium bicarbonate, pH 7.8, and part of the sample was passed through a heparin-Sepharose affinity column (Amersham Biosciences). Unbound material, the material eluted with 0.5 M NaCl, and the remaining digestion mixture were desalted with ZipTip reversed phase tips C4 and mC18 (Millipore Corp.) in tandem prior to analysis by MALDI-TOF mass spectrometry. On the basis of the obtained results a peptide spanning positions 1–19 of the mature domain was sequenced by using Fmoc (N-(9-fluorenylethoxycarbonyl) chemistries in a 433A peptide synthesizer (Applied Biosystems), purified, inspected by MALDI-TOF mass spectrometry, and analyzed for its heparin-binding potential by heparin affinity chromatography. The peptide, designated as peptide 1–19, was also analyzed by CD under similar conditions as rNC4.

Using synthetic oligonucleotides and the QuikChange™ site-directed mutagenesis kit (Stratagene), a construct was prepared for expression of mutant rNC4 carrying a sequence NGL in place of the normal sequence KRR at positions 3–5 of the polypeptide. The mutant rNC4 was expressed as above, purified using glutathione-Sepharose and Protein G, and subjected to heparin affinity chromatography.

Analysis of Disulfide Bonding Pattern—In order to isolate the NC4 domain from full-length collagen molecules, rIX was digested with collagenase (Sigma), and the reaction mixture was passed through a heparin-Sepharose affinity column (Amersham Biosciences), and the bound material, the material eluted with 0.5 M NaCl, and the remaining binding sites were blocked by injecting dilute uncoupled biotin. The low background levels from wells coated with bovine serum albumin were subtracted from the experimental data. This material and rNC4 were then subjected to trypsin digestion with or without prior reduction with DTT and alkylation with 4-vinylpyridine. For in liquid digestion, both the native and alkylated material were subjected to reversed phase chromatography on a C1 column, and the proteins were collected and dried. Trypsin (sequencing grade modified trypsin; Promega) in 50 μl of 0.1 M (NH₄)₂HCO₃, was added to the dried proteins to 5% (w/v), and digestion was carried out at 37 °C for 4 h. The digestion mixtures were analyzed by MALDI-TOF mass spectrometry.

Fourier Transform Infrared Spectroscopy—Secondary structures of rNC4 and its mutated form were analyzed by Fourier transform infrared (FTIR) spectroscopy. A sample containing rNC4 in PBS (0.137 M NaCl, 10 mM phosphate buffer, pH 7.4) was exchanged into D₂O and concentrated to about 20 mg/ml. Spectra were recorded at room temperature in IR cells having CaF₂ windows and 15-μm Teflon spacers using a Bruker I55 spectrometer and a Hg/Cd/Te infrared detector. For each sample, 15 spectra of 64 scans were averaged, and the separately collected solution background absorption for each sample in the same cell assembly was subtracted from the final spectra. Gaussian curve fitting was used to determine the secondary structures.

Mass Spectrometry—Electrospray ionization mass spectra of the expressed rNC4 were obtained using a Micromass Q-TOF quadrupole/time-of-flight hybrid mass spectrometer (Micromass) calibrated using myoglobin as a standard. Protein masses were calculated by deconvolution using the MassLynx 3.4 (Micromass).

The NC4 peptides generated by either trypsin or V8 protease digestion were analyzed with a Biflex™ MALDI-TOF mass spectrometer (Bruker-Daltonics) in positive ion reflector mode (for peptides under 3500 Da) and positive ion linear mode (for peptides larger than 3500 Da) using α-cyano-4-hydroxycinnamic acid as the matrix. The MALDI spectra were externally calibrated with the standard peptides, angiotensin I and adrenocorticotropic 18–39 or insulin.

RESULTS

Production of the Recombinant NC4 Domain of Human Collagen IX—To study the structure and interactions of the NC4 domain of human collagen IX, sequences encoding amino acids 24–268 of the human α1(IX) polypeptide were cloned into a prokaryotic expression vector for production as a C-terminal GST fusion protein lacking the native α1(IX) signal peptide. Following induction in E. coli BL21(DE3) cells, the recombinant protein was isolated from the harvested cells and subjected to initial affinity purification by glutathione-Sepharose. The GST tag was removed enzymatically, and a solution containing the suspected recombinant NC4 domain was applied to a heparin-Sepharose affinity column at physiological pH and ionic strength. A protein with a size expected for the recombinant NC4 domain bound effectively to the column and began to elute in the presence of about 0.25 M NaCl. Further purification was obtained by cation exchange chromatography. The purified protein was identified as the NC4 domain by amino acid analysis and analysis of the tryptic peptides. The expected molecular mass of 27,446 Da was obtained for the protein, designated as rNC4, by electrospray ionization mass spectrometry.

Surface Plasmon Resonance and Solid-state Analyses of the Interaction of rNC4 with Heparin—In the surface plasmon resonance analyses carried out to study the heparin-binding properties of rNC4, high molecular weight heparin was biotinylated and used to coat surface plasmon resonance sensor chips. Injection of soluble rNC4 at physiological pH and ionic strength...
strength over the heparin surface demonstrated the ability of the NC4 domain to interact with the immobilized ligand (Fig. 1). A series of injections was used to determine the monomeric binding kinetics of the heparin interaction (Fig. 2A), resulting in the determination of the association and dissociation rate constants \( k_a \) and \( k_d \) and the equilibrium dissociation constant \( K_d \) (Table I). Using a hydrophobic sensor chip surface coated with biotinylated liposomes, a \( K_d \) of 0.6 \( \mu \)M was reliably obtained with low and regenerable background levels (Table I). Relatively low \( \chi^2 \) values indicate that the Langmuir 1:1 binding model provides a good approximation of the interaction strength (Fig. 2A). Use of a carboxymethylated dextran-based sensor chip with a streptavidin coating gave an approximate \( K_d \) of 3.2 \( \mu \)M but with accumulating background levels.

Interaction of full-length rcIX with heparin was likewise analyzed using a hydrophobic sensor chip and biotinylated heparin (Fig. 2B). Using the Langmuir model, an averaged \( K_d \) of 3.6 \( \mu \)M was obtained (Table I). The use of a bivalent binding model did not result in improved fitting or a change in the relative binding level was obtained even at a 100-fold higher concentration of rNC4 than rcIX (Table I). A series of injections was used to determine the number and approximate locations of the heparin-binding sites along collagen IX, electron microscopy analysis (Fig. 3).

### Table I

| Analyte | \( k_a \) ± S.D. | \( k_d \) ± S.D. | \( K_d \) ± S.D. |
|---------|-----------------|-----------------|-----------------|
| rNC4    | 7.8 \( \times 10^{-3} \) ± 1.8 \( \times 10^{-3} \) | 4.8 \( \times 10^{-3} \) ± 0.09 \( \times 10^{-3} \) | 640 ± 150 |
| rcIX    | 140 \( \times 10^{-3} \) ± 80 \( \times 10^{-3} \) | 0.4 \( \times 10^{-3} \) ± 0.03 \( \times 10^{-3} \) | 3.6 ± 1.9 |

![Fig. 1. Binding of rNC4 to heparin, detected by surface plasmon resonance assay.](image)

![Fig. 2. Kinetics of the binding of rNC4 and rcIX to immobilized heparin, detected by surface plasmon resonance assay.](image)

![Fig. 3. Binding specificity of rNC4 with heparin, analyzed by competition with glycosaminoglycans in surface plasmon resonance assay.](image)
microscopy on negatively stained complexes of full-length collagen IX and heparin-albumin conjugated with colloidal gold was performed (Fig. 5). Results suggest the presence of four binding sites for heparin on collagen IX, one located at or near each NC domain of the protein.

Identification of the Heparin-binding Region of the NC4 Domain—Alkylated rNC4 was digested with V8 protease and the resulting mixture of peptides subjected to heparin affinity chromatography. Analysis of the peptide mixture by MALDI-TOF mass spectrometry along with unbound and bound material from the affinity chromatography revealed that the extreme N-terminal peptide (amino acids 1–19, monoisotopic mass 2098.078) was able to bind to heparin, whereas other fragments were not. The N-terminal 19-amino acid fragment was subsequently prepared as a synthetic peptide (peptide 1–19), which was shown to bind to heparin-Sepharose and elute upon introduction of about a 0.15 M concentration of NaCl (i.e. a 40% smaller concentration than was required for the elution of native rNC4). CD analysis of peptide 1–19 indicated that it was largely devoid of secondary structure (data not shown).

A mutant form of rNC4, carrying a sequence NGL in place of the basic amino acid sequence KRR of the suspected heparin-binding site, was created. Using heparin affinity chromatography, it was demonstrated that this mutant rNC4 was unable to interact with heparin at physiological conditions. Comparison of the mutant rNC4 with wild-type rNC4 by far-UV CD analysis (data not shown) and by FTIR spectroscopy (Fig. 6) showed that the mutation did not affect the secondary structure of the NC4 domain. Both the mutant and the wild-type rNC4 appear to consist mainly of β-sheet, with smaller amounts of other types of secondary structures present (Fig. 6) (21). The possibility that freezing and lyophilization would affect the structure of rNC4 was also ruled out by FTIR measurements (Fig. 6).

Circular Dichroism Analyses—CD spectrum of rNC4 was measured at 25 °C (Fig. 7A). An identical spectrum was obtained for the same sample under reducing conditions (Fig. 7B). In support of the FTIR analysis, the CD spectra at low temperatures present maximal negative ellipticity near 215 nm with no significant shoulder around 220 nm, indicating a secondary structure dominated by β-sheet. The thermal stability of rNC4 was assessed at a wavelength of 205 nm, increasing the temperature linearly from 10 to 90 °C. A midpoint temperature of thermal denaturation of about 79 °C was obtained for rNC4 (Fig. 7A, inset). In the presence of a reducing agent, this value was lowered to about 72 °C (Fig. 7B, inset). These results indicate that the notably high thermal tolerance of rNC4 is in part due to the presence of stabilizing intramolecular disulfide bonds. This conclusion was verified by analogous results indicating decreased thermal stability in a mutant form of rNC4, where Cys219 is replaced by an Arg residue (data not shown). The presence of an isodichroic point at about 193 nm (Fig. 7) indicates that the thermal unfolding occurs in a two-state transition between native and unfolded conformation.

Far-UV CD analysis of the NC4 domain isolated from full-length rIX produced in insect cells gave a similar, but not identical, spectrum as that of rNC4 (data not shown). The minor difference is probably explained by the presence of Nglycans in the material produced in insect cells, a finding that was demonstrated by results obtained in peptide mapping ex-
Far-UV spectra of 10 identical, indicating a similar secondary structure consisting mainly of lyophilized wild-type rNC4 (a), nonlyophilized wild-type rNC4 (b), and lyophilized mutated rNC4 dissolved in D$_2$O (c) are shown in the amide I region between 1700 and 1600 cm$^{-1}$. All three spectra are nearly identical, indicating a similar secondary structure consisting mainly of $\beta$-sheet, as judged by the position (~1635 cm$^{-1}$) of the main peak of the spectra.

![Image](99x287 to 266x556)

**FIG. 6.** IR spectra of rNC4 in the amide I region. The spectra of lyophilized wild-type rNC4 (a), nonlyophilized wild-type rNC4 (b), and lyophilized mutated rNC4 dissolved in D$_2$O (c) are shown in the amide I region between 1700 and 1600 cm$^{-1}$. All three spectra are nearly identical, indicating a similar secondary structure consisting mainly of $\beta$-sheet, as judged by the position (~1635 cm$^{-1}$) of the main peak of the spectra.

Experiments (data not shown) and by the heterogeneity of this material.

The structure of rNC4 was also studied by near-UV spectroscopy in the presence or absence of excess heparin. Only minimal changes in the spectrum of rNC4 were seen upon incubation with heparin, suggesting that association with heparin does not involve any major rearrangements of the tertiary structure of the NC4 domain.

Identification of the Disulfide Bonding Pattern of the NC4 Domain—In an attempt to identify the pattern of disulfide bond formation within the NC4 domain, an aliquot of rNC4 was alkylated with 4-vinylpyridine and digested with trypsin. Comparison of the pattern of the resulting peptides with that obtained by digestion of native rNC4 without alkylation indicated that all four cysteine residues of the NC4 domain appeared to be involved in disulfide bonding, with a pattern of Cys$^{21}$–Cys$^{219}$ and Cys$^{175}$–Cys$^{230}$ (Table II). An identical pattern was demonstrated for the NC4 domain isolated from full-length rcIX.

**TABLE II**

| Tryptic peptide | Amino acids | Calculated mass | Measured mass |
|-----------------|-------------|-----------------|--------------|
|                 |             | Da              | Alkylated    | Native       |
| P4$^a$          | 8–23        | 1675.762        | 1780.978$^a$|
| P6              | 26–44       | 2134.058        |              |
| P10             | 54–65       | 1234.692        | 1234.807     | 1234.842     |
| P11             | 66–73       | 933.467         | 933.680      | 933.735      |
| P13             | 78–95       | 2133.042        | 2133.308     | 2133.313     |
| P16             | 104–116     | 1574.748        | 1574.895     | 1575.016     |
| P18             | 123–135     | 1469.751        | 1469.869     | 1469.979     |
| P19             | 136–159     | 2608.256        | 2608.707     | 2608.631     |
| P20             | 160–186     | 816.453         | 816.636      | 816.770      |
| P21$^a$         | 167–177     | 1211.560        | 1316.729$^a$|
| P22             | 178–186     | 1051.639        | 1051.762     | 1051.879     |
| P23             | 187–199     | 1300.703        | 1300.807     | 1300.934     |
| P24$^a$         | 200–225     | 3073.563        |              |
| P26$^a$         | 227–235     | 1054.487        | 1159.638$^a$|
| P27             | 236–245     | 1146.552        | 1146.708     | 1146.775     |
| P4 + P24        |              | 4750.4$^d$      | 4750.6$^b$   |
| P21 + P26       |              | 2284.047        | 2284.390     |

$^a$ The mass of ethylpyridine (105.1 Da) is included.

$^b$ Average mass.

![Image](99x631 to 266x738)

**FIG. 7.** CD spectra of rNC4 as a function of temperature. Far-UV spectra of 10 μM rNC4 under nonreducing (A) and reducing conditions (B) were measured for the 185–250-nm region at temperatures ranging from 5 to 90 °C in steps of 5 °C. Results obtained at selected temperatures are shown as indicated. Thermal stability of rNC4 was also studied by monitoring the CD signal at 205 nm upon heating the sample at a rate of 0.5 °C/min under nonreducing (inset of A) and reducing (inset of B) conditions. Results show that upon reduction, the midpoint temperature of thermal transition of rNC4 was lowered by about 7 °C.

To analyze the interaction of COMP in a more reliable one-to-one situation, we used a biotinylated recombinant 57-kDa C-terminal fragment of the COMP monomer (COMP T3+TC) to coat a hydrophobic sensor chip. Injection of soluble pentameric rCOMP (11) over the surface at various concentrations resulted in typical concentration-dependent association and dissociation curves (Fig. 8A), but there seemed to be concentration-dependent differences in the kinetics of the interaction that would require further analysis. Also, treatment with EDTA did not result in complete regeneration of the surface, and therefore the resulting $K_d$ values in the subnanomolar range appear not to be fully reliable, despite the low $\chi^2$ values obtained with the Langmuir 1:1 model. The use of bivalent binding model did not improve the quality of the curve fitting further.

To analyze the interaction of COMP T3+TC with rNC4 and rcIX, Heparin was found to inhibit both interactions at low
micromolar concentrations. Preincubation of 20 nM rcIX with 4 μM heparin (molecular mass 6 kDa) prior to injection over the coated surface resulted in more than 99% inhibition of the interaction with COMP T3+TC, and 50% inhibition was achieved with a heparin concentration lower than 0.1 μM (Fig. 8). The kinetic parameters were obtained with the Langmuir 1:1 model. The y² values representing the S.E. of residuals of the fitting are shown next to each pair of curves. B, biotinylated COMP T3+TC was bound to a biotinylated lipid surface on an HPA sensor chip via NeutrAvidin™, and rNC4 was injected over the surface at concentrations of 3.55 μM (a), 7.1 μM (b), 10.65 μM (c), and 14.2 μM (d), using a 2-min contact time and a flow rate of 40 μl/min. The level of bound rcIX was read 30 s after the injection. The respective response level of the negative control channel was subtracted from that of the COMP T3+TC channel obtained for each heparin concentration (40 nM, 0.1 μM, 0.4 μM, and 4 μM), and relative binding levels were calculated using the response level obtained by injecting rcIX without heparin as a maximum.

Our results show that the NC4 domain can now be added to the growing list of extracellular matrix proteins that interact with heparin or heparan sulfate. Pepsin-treated collagen IX, together with several other collagens residing in cartilage, is known to interact with heparin (9). Collagen XI, for example, possesses several heparin-binding sequences, two of which comply with the heparin-binding consensus sequence XBBXB, where B denotes a basic amino acid and X is any other amino acid (25). A sequence KRRPRF matching this consensus is present in the extreme N-terminal region of the NC4 domain of collagen IX and is indeed shown here to be the site of interaction of the domain with heparin. This sequence motif is also present in the N terminus of the mouse collagen α1(IX) chain (26). Comparison of this sequence of NC4 with a reported distribution of residues in known heparin-binding proteins (27) further implicates the sequence as a potential heparin-binding site. Since a synthetic peptide representing the 19 extreme N-terminal amino acids of the NC4 domain elutes from a heparin affinity column with a buffer of lower ionic strength than the full-length NC4 domain, it seems that a proper structural context is required in addition to the consensus binding site in order to obtain the full heparin affinity of the NC4 domain. In support, CD analysis of the peptide indicated a significant lack of secondary structure.

The electron microscopic analysis of collagen IX complexed with heparin verifies the previously suggested presence of at least one heparin-binding site on collagen IX outside its NC4 domain (9). Our results suggest the presence of such a site at or near each NC domain of the protein. These regions of the collagen IX polypeptides do not contain any basic sequence stretches matching the linear consensus sequence (25). However, basic residues with apparent spacings of about 20 Å, an arrangement found at the heparin-binding sites of some collagens and many other proteins (25, 28), are present at the NC2 domain and at the COL2 domain not far from the NC3. In addition, most of these residues are highly conserved both between different α chains and between mammalian and avian species. As in other proteins (28), a few basic residues are found amid the ones spaced at 20-Å intervals but show somewhat less conservation. In the NC1 domain, the linear spacing of the basic residues at 7- or 8-residue intervals is less apparent, although a cluster of at least 5 basic residues is found in the C-terminal half of the human, mouse, and chicken NC1 domains. We have shown that the NC4 domain of collagen IX is clearly capable of interaction with heparin in vitro at physiological pH

FIG. 8. Kinetics of the interaction of COMP with rNC4, analyzed by a surface plasmon resonance assay. A, carboxyl-biotinylated rNC4 was bound to a biotinylated lipid surface on an HPA sensor chip via NeutrAvidin™, and rCOMP was injected over the surface at concentrations of 0.5 nM (a), 2.5 nM (b), 10 nM (c), and 25 nM (d), using a 10-min contact time and a flow rate of 10 μl/min. The experimental signals (black curves) are shown with the fits (gray curves) calculated with the Langmuir 1:1 model. Y² values representing the S.E. of residuals of the fitting are shown next to each pair of curves. B, biotinylated COMP T3+TC was bound to a biotinylated lipid surface on an HPA sensor chip via NeutrAvidin™, and rNC4 was injected over the surface at concentrations of 3.55 μM (a), 7.1 μM (b), 10.65 μM (c), and 14.2 μM (d), using a 2-min contact time and a flow rate of 40 μl/min. The level of bound rcIX was read 30 s after the injection. The respective response level of the negative control channel was subtracted from that of the COMP T3+TC channel obtained for each heparin concentration (40 nM, 0.1 μM, 0.4 μM, and 4 μM), and relative binding levels were calculated using the response level obtained by injecting rcIX without heparin as a maximum.

FIG. 9. Surface plasmon resonance analysis of heparin competition with the interaction of rcIX with COMP T3+TC. Biotinylated COMP T3+TC was bound to an HPA sensor chip, and 20 nM rcIX with or without added heparin was injected over the surface using a 60-s contact time and a flow rate of 40 μl/min. The level of bound rcIX was read 30 s after the injection. The respective response level of the negative control channel was subtracted from that of the COMP T3+TC channel obtained for each heparin concentration (40 nM, 0.1 μM, 0.4 μM, and 4 μM), and relative binding levels were calculated using the response level obtained by injecting rcIX without heparin as a maximum.
and ionic strength, albeit with a relatively low affinity. This affinity may be significant in vivo, however, since other components are likely to be involved. Collagen IX is known to interact with COMP via its NC domains (11, 12), and structural alterations in these two proteins can lead to a similar phenotype, multiple epiphyseal dysplasia, MED (13). In addition, collagen IX reportedly interacts with matrilin-3, which is yet another protein implicated in the pathogenesis of MED (13). Here we demonstrated an interaction between rNC4 and the C-terminal fragment of COMP with a $K_d$ of 230 nM, whereas a $K_{D,0}$ value of 71 nM was recently obtained by a solid-state assay using rIX instead of rNC4 (16). The difference in the magnitude for rIX and rNC4 is most likely explained by the presence of three additional COMP-binding sites in rIX, although other differences in the experimental setup may also have contributed. On the basis of our results, it is impossible to estimate whether the affinity of the NC4 for COMP is in any way affected by the absence or presence of the adjacent collagen IX triple helix. In an earlier study, an approximate $K_d$ of 32 nM was obtained for the interaction of rIX with pentameric rCOMP (11). In light of the recent results (16), our observation of a clearly lower approximate $K_d$ of 0.9 nM toward rIX when using COMP T3+TC, instead of rCOMP, appears as an underestimation, but it may also reflect the different technical approach used here.

Somewhat surprisingly, our results show that COMP and heparin compete for the same binding site or overlapping ones in the NC4 domain. Similarly, we were able to block the interaction of full-length rIX with the C-terminal domain of COMP using relatively low heparin levels. It is unclear whether these findings translate into a mechanism for controlling the interaction of collagen IX with COMP in vivo by means of heparan sulfate proteoglycans or whether they are an indication of two distinct functional roles for the NC4 domain in different regions of the cartilage extracellular matrix.

Using full-length recombinant collagen IX, we obtained an apparent $K_d$ of 3.6 nM for its interaction with heparin. Pepsin-treated collagen IX has previously been shown to bind heparin with a $K_d$ of 7 nM (9). Both of these figures are about 2 orders of magnitude smaller than the $K_d$ obtained here for the interaction of NC4 with heparin in vitro. As suggested previously (9) and verified here, additional heparin-binding sites are present in the long helical arm of collagen IX outside the NC4 domain, explaining the difference in $K_d$ for NC4 and full-length collagen IX. Unfortunately, no experimental data are available regarding the affinities of the different collagen IX heparin-binding sites for heparan sulfate proteoglycans in situ. Collagen IX lies parallel to the surface of the collagen fibril in the cartilage extracellular matrix and is covalently attached to it via the long arm (3), and therefore the accessibility of the other binding sites may be sterically reduced, leading to preferred binding of heparan sulfate proteoglycans to the NC4 domain of collagen IX.

The functional significance of the heparin-binding potential of the NC4 domain remains unknown. Interestingly, in addition to mutations affecting the COL3 domain of collagen IX, a variant of MED can also be caused by a homozygous mutation in the gene encoding the diastrophic dysplasia sulfate transporter, possibly as a result of incomplete sulfation of proteoglycans (29). Cartilage extracellular matrix reportedly contains some heparan sulfate proteoglycans (e.g. perlecain (30)), which are potential interaction partners for collagen IX. Such small proteoglycans may fulfill a bridging function between adjacent collagen IX-containing fibrils. This would explain the importance of collagen IX in the maintenance of the long term integrity of the fibrillar network (4, 5). It has been suggested that the homologous collagens XII and XIV provide a similar bridging mechanism via their NC-terminal domains, and there is some evidence that these domains modulate the biomechanical properties of tissues by controlling the organization of the fibrillar network (31, 32). Another function for the collagen IX heparin-binding potential can also be hypothesized, however, since chondrocytes are known to express cell surface heparan sulfate proteoglycans (e.g. syndecan-1, -2, and -4 (33, 34)), which are believed to contribute to integrin-mediated cell attachment and signaling (35). Despite the low affinity of NC4 for heparin in vitro, collagen IX may interact with such a cell surface receptor in vivo if other components participate in the macromolecular complex. Of the other homologous proteins, collagen XIV has been shown to bind several cell types in a heparin-inhibitable fashion via the NC-terminal noncollagenous domain of the molecule (36, 37), and heparin is also involved in the attachment of chondrocytes to collagen XI in vitro (25). Chondrocyte survival and differentiation, on the other hand, are known to be mediated by integrins in situ (38), but integrins are thought to require a co-receptor for full activity (35). It has been demonstrated recently that chondrocytes require the presence of collagen fibrils with suitable suprastructures for maintenance of the cartilage phenotype (39). Taking the above information together, it can be hypothesized that a macromolecular complex including collagen IX, a cell surface heparan sulfate proteoglycan, and perhaps other macromolecules may act in concert with the integrin system and serve as a mechanism that provides the chondrocyte with the ability to accommodate to or resist changes in the surrounding extracellular matrix. A structural alteration in any component of this complex would compromise the integrity of the cartilage. This hypothesized role of collagen IX in cell attachment and/or signaling is in harmony with the known preferential location of collagen IX in the thinnest fibrils (40, 41) that prevail in the pericellular basket surrounding the chondrocytes (42, 43). Direct experimental evidence of the participation of collagen IX in the macromolecular complexes suggested above is, however, of utmost importance to validate any of the above hypotheses.

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REFERENCES

1. van der Rest, M., and Mayne, R. (1987) in Structure and Function of Collagen Types (Mayne, R., and Burgesson, B. E., eds) pp. 185–221, Academic Press, Inc., Orlando, FL.
2. Mayne, R., and Brewton, R. G. (1993) Curr. Opin. Cell Biol. 5, 883–890
3. Eyre, D. (2002) Arthritis Res. 4, 30–35
4. Olsen, B. R. (1997) Int. J. Biochem. Cell Biol. 29, 555–558
5. Aszodi, A., Bateman, J. F., Gustafsson, E., Boot-Handford, R., and Fassler, R. (2000) Cell Struct. Funct. 25, 73–84
6. Irwin, M. H., Silvers, S. H., and Mayne, R. (1985) J. Cell Biol. 101, 814–823
7. Vaughan, L., Menderl, M., Huber, S., Bruckner, P., Winterhalter, K. H., Irwin, M. H., and Mayne, R. (1988) J. Cell Biol. 106, 991–997
8. Vasios, G., Nishimura, I., Konomi, H., van der Rest, M., Ninomiya, Y., and Olsen, B. R. (1988) J. Biol. Chem. 263, 2324–2329
9. Munakata, H., Takagaki, K., Majima, M., and Endo, M. (1999) Glycobiology 9, 1023–1027
10. Bork, P. (1992) FEBS Lett. 307, 49–54
11. Thrur, J., Rosenberg, K., Nitsche, D. P., Pihlajamaa, T., Ala-Kokko, L., Heinegard, D., Paulsson, M., and Maurer, P. (2001) J. Biol. Chem. 276, 6083–6092
12. Holdén, P., Meadows, R. S., Chapman, K. L., Grant, M. E., Kadler, K. E., and Briggs, M. D. (2001) J. Biol. Chem. 276, 6046–6055
13. Briggs, M. D., and Chapman, K. L. (2000) Hum. Mutat. 19, 465–478
14. Pihlajamaa, T., Peralta, M., Vuorio, M. M., Nokelainen, M., Bodo, M., Schultzess, T., Vuorio, E., Timpl, R., Engel, J., and Ala-Kokko, L. (1999) J. Biol. Chem. 274, 23464–23468
15. Douglas, S. P., Jenkins, J. M., and Kadler, K. E. (1998) Matrix Biol. 16, 497–505
16. Spitsnagel, L., Nitsche, D. P., Paulsson, M., Maurer, P., and Zaucke, F. (2004) Biochim. Biophys. Acta 1744, 479–487
17. Cooper, M. A., Hansson, A., Lofås, S., and Williams, D. H. (2000) Anal. Biochem. 277, 196–205
18. Brown, R. G. (1993) J. Cell Biol. 122, 1069–1078
19. Bork, P. (1992) FEBS Lett. 307, 49–54
20. Thrur, J., Rosenberg, K., Nitsche, D. P., Pihlajamaa, T., Ala-Kokko, L., Heinegard, D., Paulsson, M., and Maurer, P. (2001) J. Biol. Chem. 276, 6083–6092
21. Holdén, P., Meadows, R. S., Chapman, K. L., Grant, M. E., Kadler, K. E., and Briggs, M. D. (2001) J. Biol. Chem. 276, 6046–6055
22. Briggs, M. D., and Chapman, K. L. (2000) Hum. Mutat. 19, 465–478
23. Pihlajamaa, T., Peralta, M., Vuorio, M. M., Nokelainen, M., Bodo, M., Schultzess, T., Vuorio, E., Timpl, R., Engel, J., and Ala-Kokko, L. (1999) J. Biol. Chem. 274, 23464–23468
24. Douglas, S. P., Jenkins, J. M., and Kadler, K. E. (1998) Matrix Biol. 16, 497–505
25. Spitsnagel, L., Nitsche, D. P., Paulsson, M., Maurer, P., and Zaucke, F. (2004) Biochim. Biophys. Acta 1744, 479–487
26. Cooper, M. A., Hansson, A., Lofås, S., and Williams, D. H. (2000) Anal. Biochem. 277, 196–205
18. Pignataro, B., Steinem, C., Galla, H. J., Fuchs, H., and Janshoff, A. (2000) *Biophys. J.* 78, 487–498
19. Rosenberg, K., Olson, H., Mørgelin, M., and Heinegård, D. (1998) *J. Biol. Chem.* 273, 20397–20403
20. Wiberg, C., Klatt, A. R., Wagener, R., Paulsson, M., Bateman, J. F., Heinegård, D., and Mørgelin, M. (2003) *J. Biol. Chem.* 278, 37698–37704
21. Pelto, J. T., and McLean, L. R. (2000) *Anal. Biochem.* 277, 167–176
22. Bruckner, P., Mayne, R., and Tuderman, L. (1983) *Eur. J. Biochem.* 136, 333–339
23. Miles, C. A., Knott, L., Sumner, I. G., and Bailey, A. J. (1998) *J. Mol. Biol.* 277, 135–144
24. Baneyx, F. (1999) *Curr. Opin. Biotechnol.* 10, 411–421
25. Vaughan-Thomas, A., Young, R. D., Phillips, A. C., and Duance, V. C. (2001) *J. Biol. Chem.* 276, 5303–5309
26. Rokos, I., Muragaki, Y., Warman, M., and Olsen, B. R. (1994) *Matrix Biol.* 14, 1–8
27. Cardin, A. D., and Weintraub, H. J. R. (1999) *Arteriosclerosis* 9, 21–32
28. Margalit, H., Fischer, N., and Ben-Sasson, S. A. (1995) *J. Biol. Chem.* 268, 19228–19231
29. Russi, A., and Superti-Furga, A. (2001) *Hum. Mutat.* 17, 159–171
30. SundarRaj, S., Fite, D., Ledbetter, S., Chakravarti, S., and Hassell, J. R. (1995) *J. Cell Sci.* 108, 2663–2672
31. Nishiyama, T., McDonough, A. M., Bruns, R. R., and Burgeson, R. E. (1994) *J. Biol. Chem.* 269, 28193–28199
32. Akutsu, N., Milbury, C. M., Burgeson, R. E., and Nishiyama, T. (1999) *Exp. Dermatol.* 8, 17–21
33. Barre, P. E., Redini, F., Boumediene, K., Vialleau, C., and Pujol, J.-P. (2000) *Osteoarthritis Cartilage* 8, 34–43
34. Pflander, D., Svoboda, B., and Kirsch, T. (2001) *Am. J. Pathol.* 158, 1777–1783
35. Couchman, J. R., and Woods, A. (1999) *J. Cell Sci.* 112, 3415–3420
36. Ehni, T., Dieterich, W., Bauer, M., von Lampe, B., and Schuppan, D. (1996) *Exp. Cell Res.* 229, 388–397
37. Ehni, T., Dieterich, W., Bauer, M., and Schuppan, D. (1998) *Exp. Cell Res.* 239, 477–480
38. Hirsch, M. S., Lunsford, L. E., Trinkaus-Randall, V., and Svoboda, K. K. (1997) *Dev. Dyn.* 210, 249–263
39. Farjanel, J., Schurmann, G., and Bruckner, P. (2001) *Osteoarthritis Cartilage* 9, Suppl. A, 55–63
40. Hagg, R., Bruckner, P., and Hedbom, E. (1998) *J. Cell Biol.* 142, 285–294
41. Poole, C. A., Flint, M. H., and Beaumont, B. W. (1987) *J. Orthop. Res.* 5, 509–522
42. Hunziker, E. B., Michel, M., and Studer, C. (1997) *Micros. Res. Tech.* 37, 271–284
Characterization of Recombinant Amino-terminal NC4 Domain of Human Collagen IX: INTERACTION WITH GLYCOSAMINOGLYCANS AND CARTILAGE OLIGOMERIC MATRIX PROTEIN

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