Alternative Splicing of the First F3 Domain from Chicken Collagen XIV Affects Cell Adhesion and Heparin Binding*

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The N terminus of chicken collagen XIV is subject to alternative splicing. The longer isoform contains a fibronectin type III (F3) domain at its N terminus, whereas the shorter isoform is lacking this domain. Alternative splicing of the F3 domain is developmentally regulated. At early embryonic stages, both isoforms are expressed, whereas after hatching only the longer isoform is expressed. When immobilized on plastic dishes, the recombinant F3 domain promotes the adhesion of mesenchymal cells. Attachment to this domain is specifically inhibited by heparin but not by other glycosaminoglycans. Molecular modeling studies illustrate that the first F3 domain harbors a positively charged groove, which may accommodate the negatively charged heparin chain. Site-directed mutagenesis of a single lysine residue within this groove abolishes the cell binding activity but does not affect the heparin binding activity. Cell binding and heparin binding are therefore two functionally distinct properties shared by the N-terminal F3 domain. When full-length collagen XIV polypeptides that either contain or lack the first F3 domain are tested on heparin-Sepharose, a pronounced difference in their relative affinity is observed. Thus, alternative splicing of the N-terminal F3 domain influences the interaction of this FACIT (fibril-associated collagens with interrupted triple helices) collagen with cells and with glycosaminoglycans.

The biochemical properties of interstitial collagen fibrils are modulated by a distinct family of adapter proteins, which add new functional domains to the fibrils and which may control their precise architecture. This family is represented by the fibril-associated collagens with interrupted triple helices (FACIT) and includes collagens IX, XII, XIV, XVI, and XIX (1, 2). Alternative splicing of the first F3 domains influences the interaction of these FACIT molecules with interrupted triple helices (F3) domains of other collagens (3-6). This homotrimolecular glycoprotein is expressed in every tissue that contains type I collagen (1, 5, 6). It is associated with the surface of interstitial collagen fibrils (7, 8), although a direct interaction with type I collagen could not be demonstrated (1, 9, 10). The complete primary structure of chicken collagen XIV has been established by cDNA cloning (4, 11). These studies revealed a protein with two short collagenous helices (COL1 and COL2) and three noncollagenous domains (NC1, NC2, and NC3). The extended, N-terminal NC3 domain makes up the majority of the entire protein (80%), whereas the two collagenous helices contribute only 14% to the molecular mass. The NC3 domain has a complex, modular structure comprising eight F3 modules related to fibronectin, two VA domains related to von Willebrand factor, and one TspN domain related to thrombospondin. Undulin, a large glycoprotein isolated from human placenta, has recently been identified as the human homologue of chicken collagen XIV (3, 7, 12, 13).

Collagen XIV interacts with a broad palette of extracellular matrix molecules and a few of these interactions have been assigned to a particular domain of the polypeptide chain. Collagen XIV binds to collagen VI (9). This interaction seems to be accomplished by the collagenous domain, because the triple helices of collagens VI and XIV have about the same length, allowing a lateral aggregation. Collagen XIV binds to procollagen N-proteinase (14). In this way the proteinase is immobilized in close vicinity to type I collagen fibrils, where it could control the processing and incorporation of newly synthesized molecules. Collagen XIV also interacts with glycosaminoglycans and proteoglycans (9). Two binding sites have been described for heparin, one in the C-terminal NC1 domain and one at the opposite end in the N-terminal NC3 domain. Although the interaction with the NC1 domain has been characterized in great detail (15, 16), not much is known about the interaction with the NC3 domain except that it is facilitated by a stretch of basic amino acids located between the first F3 repeat and the adjacent VA domain (17). Collagen XIV also interacts with the small proteoglycan decorin (18, 19), and it is likely that the two binding sites identified for heparin are also involved in this interaction.

Collagen XIV has the potential to bind to cell surfaces. In vitro, it promotes the adhesion of various mesenchymal and epithelial cells to plastic dishes. For human fibroblasts, a chondroitin/dermatan sulfate form of CD44 has been proposed as a cell surface receptor (20). Another study demonstrated adhesion of hematopoietic cells to collagen XIV and suggested a heparan sulfate proteoglycan as cell surface receptor (21). In contrast to these reports, an earlier study did not find any interaction between collagen XIV and nine different cell lines (9).

The apparent discrepancies between different reports about the interaction of collagen XIV with cells might be explained, at least in part, by the existence of different protein isoforms. Several isoforms that are likely to be generated by alternative splicing have been described for chicken collagen XIV. We...
Alternative Splicing of Chicken Collagen XIV

| No. | Sequence (5' → 3') | Positions | Purpose |
|-----|-------------------|-----------|---------|
| P1  | GCATACTGCACTCAGTTCAAG | 1807–1828 | RT      |
| P2  | ATTCAGAGATGCTGATACATCATCAAGTGG | 286–306 | RT-PCR  |
| P3  | ATCAGATGCAACATATTGCTG | 1380–1401 | RT-PCR  |
| P4  | TCACCAGTACCACGCGGCCGCCCA | 380–389 | F3-GST  |
| P5  | GCATTACCAGTGGTATGTCCTCTGT | 677–707 | F3-GST  |
| P6  | CTGGCTGCAGGCTGAGATGTGT | 729–759 | VA-GST  |
| P7  | GAACTTCCCGGTCATCCTGT | 1334–1360 | F3 + VA-GST |
| P8  | GGCAGAATCTCCACGGAGAAGATGTT | 2161–2185 | F3(5)-GST |
| P9  | GCGAGTGTGGCAATTTGAGCTCTC | 2440–2465 | F3(3)-GST |
| P10 | CTGAGACCTGACGCGCTTACCA | 3033–3063 | F3(6)-GST |
| P11 | TGCCGTCCGCTCCCGAATT | 3339–3363 | F3(6)-GST |
| P12 | CCACCAACACCTTAAATGACGTCA | 406–435 | mut35+37 |
| P13 | CCACCAAAAGCTTAAATGACGTCA | 406–435 | mut35 |
| P14 | CCACCAAAGGCTTAAATGACGTCA | 406–435 | mut37 |
| P15 | GGCACTGCGCCGCGCCGCT | 389–405 | mut35, mut37 |
| P16 | ATCTCCTGGAGCCCACCAGGGCAATTC | 454–483 | mut51+54 |
| P17 | ATCTCCTGGAGCCCACCAGGGCAATTC | 454–483 | mut51 |
| P18 | ATCTCCTGGAGCCCACCAGGGCAATTC | 454–483 | mut54 |
| P19 | pCTGCGAATGCATTGGAGCTTGCTTAAT | 424–453 | mut51, mut54 |

Oligonucleotide primers used in this study

Nucleotides that do not correspond to the original cDNA sequence (4) are underlined. Such changes were introduced into the primers to create additional restrictions sites, to mutate the codons for individual amino acids, or to enable annealing to a vector sequence.

Affinity chromatography on GST-Sepharose. If required, the GST moiety was released from the fusion protein by cleavage with endopeptidase factor Xa (Roche Molecular Biochemicals) and removed by adsorption to GSH-Sepharose.

**Site-directed Mutagenesis**—The codons for individual amino acids of the first F3 domain were mutated by the ExSite PCR-based mutagenesis method (Stratagene). The forward primer harbored the desired mutation, while the reverse primer was phosphorylated at its 5′-end and selected in a way that it annealed directly adjacent to the 5′-end of the forward primer (Table I). To improve the efficiency of amplification, the template cDNA was cloned into pBluescript SK+ and all mutations were performed in this relatively small plasmid. After amplification through 21 cycles of 1′ at 95 °C, 2′ at the optimal annealing temperature of the primer pair used (54–62 °C), and 8′ at 72 °C, the maternal DNA was removed by digestion with the restriction enzyme DpnI (Roche Molecular Biochemicals). The ends of the linear products were joined by ligation with T4 DNA ligase (Roche Molecular Biochemicals), and the circular, nicked plasmids were transfected into competent bacteria (E. coli XL-1 blue). Authenticity and reading frame of all mutated clones were verified by DNA sequencing. Finally, the inserts with the desired mutation were subcloned into the EcoRI/XhoI site of the expression vector pGEX-5X and expressed in E. coli BL21 as described above.

**Cell Culture**—Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA): U266 (TIB-196), U937 (CRL-1593.2), KG-1a (CCL-246.1), HT1080 (CCL-121). Primary chicken fibroblasts (CTF) and primary chicken smooth muscle cells (CSM) were prepared from tendons and gizzards of 17-day-old chicken embryos with the help of collagenase (Roche Molecular Biochemicals). The ends of the linear products were joined by ligation with T4 DNA ligase (Roche Molecular Biochemicals), and the circular, nicked plasmids were transfected into competent bacteria (E. coli XL-1 blue). Authenticity and reading frame of all mutated clones were verified by DNA sequencing. Finally, the inserts with the desired mutation were subcloned into the EcoRI/XhoI site of the expression vector pGEX-5X and expressed in E. coli BL21 as described above.

**Cell Adhesion Experiments**—Two different assays were used to determine the cell binding activity of GST fusion proteins. For the method of Klein et al. (21), water droplets (5 µl) containing 10 pmol of fusion protein were spotted onto virgin plastic plates (Falcon 1007, 60-mm diameter) and air-dried. Residual binding sites of the plates were blocked with bovine serum albumin (10 mg/ml in RPMI 1640 medium). Cells were seeded onto the washed plates (105 cells/plate) and allowed to attach for 1 h at 37 °C. Nonadherent cells were removed by three gentle washing steps with phosphate-buffered saline, and adherent

reported the existence of two splice variants that possess distinct C-terminal domains (4). These variants differ by the inclusion or skipping of 93 nucleotides encoding 31 amino acids of the NC1 domain. Another splice variant concerns the sixth F3 repeat in the center of chicken collagen XIV. This F3 repeat either contains or lacks an insert of the three amino acids Val-Arg-Thr (22). Two additional transcripts with alternative 5′-regions have been identified by Gerecke et al. (11). Because these transcripts differ only in the 5′-untranslated region, they encode the same polypeptide.

A striking variation is also observed at the N terminus of the two sequences published for chicken collagen XIV. Compared with our cDNA sequence (4), the sequence reported by Gerecke et al. (11) lacks a portion of −350 bp coding for an F3 domain. So far it is not known whether this difference is simply a cloning artifact or whether it occurs under physiological conditions. We therefore set out to investigate whether the two variants are the result of alternative splicing and whether the encoded polypeptides have distinct biological properties.

**EXPERIMENTAL PROCEDURES**

**RT-PCR—Poly(A)+ RNA** was isolated from various embryonic and adult chicken tissues by the guanidinium thiocyanate method as described previously (22). The RNA was transcribed into cDNA with avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) and primer P1 (see Table I). The single-stranded cDNA was amplified by PCR through 36 cycles of 1′ at 95 °C, 2′ at 50 °C and 3′ at 72 °C using Phusion polymerase (Stratagene) and primers P2 and P3. The products were resolved on a 1% agarose gel stained with ethidium bromide. Bands of interest were subcloned into the plasmid pcDNA3.1 (Invitrogen) employing the XbaI restriction site of the forward primer P2 and the internal EcoRI site of the PCR product (position 1285 according to Ref. 4). The DNA sequence of the insert was determined by the dodeoxy chain termination method with Sequenase 2.0 (U. S. Biochemical Corp.).
cells were inspected under the microscope.

For the second method described by Ehnis et al. (20), the wells of a 96-well microtiter plate were coated with fusion proteins (1 μg/100 μl) for 4 h at 37 °C. Remaining sites were blocked with bovine serum albumin (10 mg/ml in RPMI 1640 medium). Approximately 10⁵ cells in 100 μl of RPMI 1640 medium were added to each well and, after an incubation period of 1 h at 37 °C, nonadherent cells were washed off with RPMI 1640 medium. The wells were subsequently incubated at 37 °C with 50 μl of a solution containing 3.75 mM p-nitrophenol-N-acetyl-β-p-glucosaminide, 50 mM sodium citrate, 0.25% Triton X-100, pH 5. After 40–90 min, depending on the cell line used, the reaction was stopped by addition of 100 μl of 50 mM glycine, 5 mM EDTA, pH 10.4, and the absorbance was read at 405 nm.

In some experiments the coated plastic plates were incubated with various glycosaminoglycans (0.01–100 μg/ml), heparin, heparan sulfate, and decorin from Sigma; chondroitin sulfate and hyaluronic acid from Fluka) prior to the addition of cells.

Molecular Modeling—The three-dimensional structure of several F3 domains was modeled with the help of a computer using the Swiss-Model Version 1.1 program (26). The tenth F3 domain of fibronectin was used as template (27). Homology modeling, superposition and energy minimization were done as previously described (22).

Circular Dichroism and Fluorescence Spectroscopy—The circular dichroism spectrum of purified F3 domains was recorded over a range of 190–250 nm in 50 mM sodium phosphate buffer, pH 7.5 (10 μg/ml). For fluorescence measurements, the proteins (5 μg/ml) were analyzed in a luminescence spectrometer set at 280-nm excitation and 290- to 400-nm emission. These experiments were kindly performed by Drs. T. Kiehaber and O. Bieri (University of Basel).

Full-length Constructs—Two different full-length constructs were prepared using the original cDNA clones 113, 8, and 9 (3–4) as well as the two XbaI/EcoRI fragments that either contain or lack the sequence of the first F3 domain as outlined above (RT-PCR). The final constructs harbored the following fragments in pCDNA3.1(−)· XbaI/EcoRI (positions 286–1283) derived from the two RT-PCR products; EcoRI/BetEII (positions 1283–2655) from clone 113; BetEII/PstI (positions 2655–4944) from clone 9; PstI/EcoRI (linker) (positions 4944–6558) from clone 8. Sequences of the constructs were verified by DNA sequencing with the help of several synthetic primers. The two constructs were expressed in a coupled transcription/translation system (Promega) following the instructions of the manufacturer. A typical reaction (total volume, 25 μl) contained 12.5 μl of reticulocyte lysate, 1 μl of TnT reaction buffer, 0.5 μl of T7 RNA polymerase (15 units/ml), 0.5 μl of amino acid mix (1 mM), 2 μl of [35S]methionine (1000 Ci/mmol, 10 μCi), 0.5 μl of DNA (40,000 units/ml), and 2 μg of template DNA. After incubation at 30 °C for 2 h, the reaction mixture was analyzed on an SDS-polyacrylamide gel.

Heparin Binding Studies—Bacterially expressed fusion proteins as well as in vitro translated full-length constructs were tested for their interaction with heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) in 20 mM Tris-HCl, pH 7.4. The protein solutions (100 μg of fusion protein or 75 μl of translation mixture) were applied at room temperature to the equilibrated column (bed volume 500 μl) and eluted stepwise with Tris buffer containing 0, 100, 200, 300, 400, 600, 800, 1000, 1500, and 2000 mM NaCl. The NaCl concentration of the effluent was determined with a CDMS conductivity meter (Radiometer, Copenhagen), and the radioactivity was monitored with a Tri-Carb liquid scintillation counter (Canberra Packard). Proteins in the effluent were analyzed by SDS-polyacrylamide gel electrophoresis. The gels were either processed for immunoblotting with anti-GST antibodies or dried and exposed to Kodak MR x-ray film (Rochester, NY).

RESULTS

Alternative Splicing of the N-terminal F3 Domain—Comparison of the two cDNA sequences available for chicken collagen XIV revealed a striking difference at the 5’-end. The sequence of Walchli et al. (4) contained a segment of 349 bp inserted after the sequence corresponding to the signal peptide (Fig. 1). This segment coded for 116 amino acids forming an F3 domain and a short linker. In the sequence of Gerecke et al. (11) this insert was missing and the region of the signal peptide was directly followed by the VA domain.

Because the inserted segment corresponded to a complete protein module and the reading frame was maintained, it seemed likely that the two forms were generated by an alternative splicing event. We therefore analyzed the exon/intron structure of the collagen XIV gene at this region. So far the chicken gene has not yet been isolated and characterized, but the human gene has been sequenced to a great extent in the course of the human genome project. We therefore analyzed the exon/intron structure of the human gene. This gene is located on chromosomal band 8q23 (28), which is covered to a large extent by a BAC clone (GenBank accession number AC020603) and by several GSS tags (GenBank accession numbers AQ808761, AQ466836, and AQ700646). The unrefined sequences of these clones demonstrated that the first F3 domain is encoded by two separate exons that are followed by a short exon for the linker and by four exons for the VA domain (Fig. 1C and Table I). All splice boundaries conform to the ag/gt rule. The 348-bp insert exactly spans exons 2, 3, and 4, lending strong support to the notion that the two isoforms are the result of alternative splicing. All introns in this region of the gene have a length of 1–4 kb, except for the intron separating exons 4 and 5. This intron is nearly 30 kb and may contain regulatory sequences required for alternative splicing.

In an effort to verify these results for the chicken collagen XIV gene, genomic PCR was performed utilizing exon specific primers and genomic DNA from chicken fibroblasts (not shown). Introns 2 and 3 were successfully amplified, and their DNA sequences confirmed the location of the splice boundaries deduced from the human gene. Intron 4, however, could not be amplified, suggesting that this intron must also be extremely large in the chicken gene.

Tissue Expression of the Two Isoforms—The expression of the two isoforms was studied by PCR using cDNA transcribed from poly(A) RNA of various chicken tissues. The forward primer corresponded to a sequence immediately preceding the signal peptide, the reverse primer corresponded to a sequence within the second F3 repeat (see Fig. 1). A PCR product of 1115 bp was expected for the longer splice variant, whereas a product of 767 bp was expected for the shorter splice variant lacking the F3 domain and the linker. A prominent band of ~1100 bp indicative of the longer variant was obtained with RNA from eight different embryonic tissues, including skeletal muscle, heart, gizzard, skin, liver, brain, sternum, and calvaria (Fig. 2,
left). Most of the samples also contained a band of \(~800\) bp indicative of the shorter splice variant. However, the intensity of this band was considerably weaker and varied among the samples investigated. It was prominent in the sample from brain but barely detectable in the samples from sternum and calvaria. For a detailed analysis, the two PCR products were subcloned and sequenced. The sequence of the larger band was identical with that published by Waëlchi \textit{et al.} (4), the sequence of the shorter fragment was identical with that published by Gerecke \textit{et al.} (11). Thus, the difference between the two published cDNA sequences reflects the result of inclusion or skipping of three exons.

Striking differences were observed when the relative amount of the two isoforms was analyzed at different developmental stages (Fig. 2, right). In skeletal muscle and gizzard of embryonic day 10, the shorter isoform was expressed at nearly the same level as the longer isoform. In samples from adult animals, however, the shorter variant was not detected at all. Thus, the expression of the shorter variant without N-terminal F3 repeat is developmentally regulated.

Cell Adhesion to the N-terminal F3 Repeat—To search for functional differences between the two splice variants, the N-terminal F3 repeat (residues 28–124), the N-terminal VA domain (residues 146–344) as well as the combined F3+VA domains (residues 28–344) were expressed in bacteria as GST fusion proteins. The affinity-purified fusion proteins were tested for their activity to promote cell adhesion. Two different cell binding assays previously described in the literature were employed (17, 21). For a qualitative assay, the fusion proteins were spotted onto virgin plastic plates and air-dried. Control experiments demonstrated that all the three fusion proteins adsorbed equally well to the plastic surface. Cells were seeded onto the coated plastic dishes and allowed to attach. Nonadherent cells were removed by several washing steps, and adherent cells were inspected under the microscope. For a more quantitative assay, a multiwell plate was coated with the fusion proteins and a cell suspension was applied to the coated wells. After a short incubation period, nonadherent cells were washed off and adherent cells were quantified by measuring the activity of the endogenous enzyme hexosaminidase. Although the amount of protein bound to the plastic surface may differ substantially between the two methods, both assays yielded very similar results.

In a first set of experiments, six different cell types were tested, namely, chicken tendon fibroblasts (CTF), chicken smooth muscle cells (CSM), human fibrosarcoma cells (HT-1080), human plasmacytoma cells (U266), human myeloblastic cells (KG-1a), and human promonocytic cells (U937). Four of these cell types (CTF, HT-1080, U266, and KG-1a) readily adhered to the F3 fusion protein, whereas two (CSM and U937) did not bind at all (Fig. 3). The same four cell types also adhered to the F3+VA protein, but none of the cells bound to the VA fusion protein alone. Thus, the alternatively spliced F3 domain promotes attachment of several hematopoietic and fi-
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Fig. 3. Adhesion of various cells to the recombinant proteins F3, VA, and F3+VA. The purified proteins were dissolved in water, spotted onto plastic plates, and allowed to air-dry. Residual binding sites were blocked with bovine serum albumin. Cells in serum free medium were added and allowed to attach. Nonadherent cells were washed off. A, binding of three different cell types to the F3 fusion protein; B, binding of U266 cells to the fusion proteins F3, VA, and F3+VA.

broblastic cells. Adhesion to the F3 fusion protein was dependent on the correct conformation of the protein, because it was abolished when the fusion protein was heat-denatured prior to application to the plastic surface (not shown). No adhesion was observed to the GST protein alone that was lacking the F3 domain. Furthermore, the ability to promote cell adhesion was specific for the first F3 domain of collagen XIV. It was not detected with control proteins containing either the fifth or the eighth F3 domain of chicken collagen XIV fused to GST. These results demonstrate that fibroblasts and some hematopoietic cells possess specific cell surface receptors that interact with the alternatively spliced F3 domain of collagen XIV.

To test whether cell binding was modulated by glycosaminoglycans, we examined the effect of heparin, heparan sulfate, chondroitin sulfate, hyaluronic acid, and decorin. Heparin completely inhibited the adhesion of U266 cells to the F3 and the F3+VA fusion proteins when applied to the precoated dishes prior to the addition of cells (not shown). The inhibitory effect was concentration-dependent. Half-maximal inhibition was observed at ~0.93 μg/ml with U266 cells and the F3 fusion protein. Heparan sulfate, chondroitin sulfate, and hyaluronic acid showed no effect up to a concentration of 50–100 μg/ml. Likewise, decorin, which consists of a core protein (Mr 40,000), and one chondroitin/dermatan sulfate chain (Mr 60,000) had no effect when used at 1 μg/ml. At a higher concentration of 30 μg/ml, decorin partially inhibited cell binding. The interaction of collagen XIV with its cell surface receptor is therefore modulated by heparin or by a heparin-containing proteoglycan.

Site-directed Mutagenesis of the F3 Domain—To study the interaction of the alternatively spliced F3 domain with cells in detail, we decided to replace individual amino acids by site-directed mutagenesis. To determine which residues to replace, the three-dimensional structure of the N-terminal F3 domain with adjacent linker was predicted by computer modeling studies using the tenth F3 domain of human fibronectin as template. These protein modeling studies revealed a structure with four clusters of positively charged residues that formed a prominent groove, which theoretically could accommodate a negatively charged polysaccharide chain (Fig. 4). No negatively charged residues that would repel the negatively charged polysaccharide chain occurred within this region of the F3 repeat. A similar cradle-like structure has previously been proposed to be involved in the heparin-binding activity of the tenth F3 domain in fibronectin (29).

Four of the positively charged amino acids, namely Arg-35, Arg-37, Lys-51, and Lys-54, were selected for site-directed mutagenesis. The amino acids were replaced either separately or in combination by serine, which possesses an uncharged, hydrophilic side chain. The mutated fusion proteins were expressed in bacteria and tested for their cell binding activity as described above. Mutation of Lys-51 to Ser completely abolished binding of the F3 protein to U266 cells (Fig. 5). In contrast, none of the other three single amino acid mutations (Arg-35 → Ser, Arg-37 → Ser, Lys-54 → Ser) had any influence on cell binding. However, cell binding was completely abolished when two residues were mutated in combination (Arg-35 plus Arg-37 or Lys-51 plus Lys-54). Similar results were obtained with chicken fibroblasts and with KG-1α cells. Furthermore, the droplet adhesion assay and the microtiter plate/hexosaminidase assay gave comparable results. Thus, Lys-51 plays a crucial role in cell binding to the alternatively spliced F3 domain.

To exclude the possibility that the mutated F3 domain was inactive in cell binding, because it had adopted an incorrect conformation, circular dichroism and fluorescence spectroscopy experiments were performed. The wild type and the Lys-51 → Ser mutated fusion proteins were treated with endopeptidase Xa to release the GST moiety and the circular dichroism and fluorescence spectra were recorded after affinity purification. Within experimental error, identical spectra were obtained with both protein preparations (not shown). These results indicate that the Lys-51 → Ser mutation did not cause any changes in the conformation of the F3 domain, neither in the polypeptide backbone (as revealed by circular dichroism) nor in the conformation of residues in the vicinity of aromatic amino acids (as revealed by the fluorescence spectrum).

Binding of the N-terminal F3 Domain to Heparin—Because heparin inhibits the interaction of the F3 domain with cells, we also examined a direct binding of the F3 domain to heparin. The GST fusion protein as well as its mutated form (Lys-51 → Ser) were applied to a heparin-Sepharose column and eluted in a stepwise fashion with an NaCl gradient. As seen in Fig. 6, both forms of the F3 domain bound specifically to heparin and eluted as broad peaks at an ionic strength corresponding to ~500 mM salt. Within experimental error, the wild type and the mutated F3 domain showed the same elution profile. GST alone without fusion protein did not bind to the column (not shown). Thus, the alternatively spliced F3 domain interacts with both heparin and cells. The mutated F3 domain, which has lost its cell binding activity, still retains its heparin binding activity. Cell binding and heparin binding are, therefore, two functionally distinct features shared by the alternatively spliced F3 domain.

Full-length Constructs of Collagen XIV—To verify our results obtained with the isolated F3 domain in the context of the entire collagen XIV polypeptide, we prepared full-length cDNA constructs. Five of the original 19 overlapping cDNA clones for collagen XIV were assembled to yield two full-length clones, one corresponding to the longer splice variant as described by Wächli et al. (4), the other corresponding to the shorter variant...
lacking the F3 domain as described by Gerecke et al. (11). The two full-length constructs were expressed in a coupled transcription/translation system in the presence of radioactively labeled methionine. In both cases, a prominent polypeptide was obtained that migrated on a polyacrylamide gel with an apparent mobility of ~200 kDa (Fig. 7). The variant lacking the N-terminal F3 repeat (predicted molecular mass of 190 kDa) migrated slightly faster than the variant containing this repeat (predicted molecular mass of 200 kDa). Thus, the full-length polypeptides can successfully be prepared in vitro in analytical amounts. So far, however, we have not been able to prepare chemical amounts of these polypeptides, either in a prokaryotic or in a eukaryotic expression system. Curiously enough, the full-length cDNA clones appeared to be unstable even in a prokaryotic expression system.

The two full-length polypeptides translated in vitro were applied to a heparin-Sepharose column and eluted with a stepwise salt gradient as outlined above. As shown in Fig. 7, both polypeptides bound to the column but they exhibited clearly different elution profiles. The longer splice variant with the

Fig. 4. Three-dimensional structure of the first F3 domain from chicken collagen XIV as predicted by computer modeling. Charged residues are indicated in red (basic residues) and blue (acidic residues). The four residues that were selected for site-directed mutagenesis are highlighted again on the right-hand side.

Fig. 5. Cell adhesion to mutated proteins. The wells of a microtiter plate were coated with fusion proteins. Residual binding sites were blocked with bovine serum albumin. U266 cells were added in serum free medium and allowed to attach. Nonadherent cells were removed by washing. Adherent cells were quantified by measuring the activity of the enzyme hexosaminidase. The results are expressed in relation to a positive control performed with the wild type F3 protein. Each bar represents the mean and S.D. from three independent experiments. F3(5), fifth F3 domain from chicken collagen XIV; mut 35 + 37, first F3 domain with residues 35 and 37 mutated to serine.

Fig. 6. Binding of the recombinant F3 domain to heparin. The wild type F3 domain and its mutated form (mut51; Lys-51 → Ser) were applied to a heparin-Sepharose column and eluted with a stepwise NaCl gradient. Individual fractions were analyzed on a polyacrylamide gel stained with Coomassie Blue. The actual NaCl concentration of the elution was determined with a conductivity meter and is shown at the bottom. B, breakthrough.
N-terminal F3 domain eluted as a broad peak at an ionic strength corresponding to ~500 mM NaCl. The shorter splice variant without N-terminal F3 domain eluted as a sharp peak at ~150 mM NaCl. Thus, the longer splice variant has a significantly higher affinity for heparin than the shorter variant.

Finally, we tried to demonstrate binding of the full-length polypeptides to cells. For this purpose, radiolabeled translation products were incubated with a suspension of U266 cells. After washing, the radioactive material bound to the cells was solubilized in SDS sample buffer and analyzed on a polyacrylamide gel. Unfortunately, these experiments did not yield any conclusive results. The cells did not bind significantly more radioactivity than background controls, and the bound material, when analyzed on polyacrylamide gels, did not migrate any more in the 200-kDa region, suggesting that unspecific degradation had occurred.

**DISCUSSION**

Several recent reports provide compelling evidence that chicken collagen XIV occurs in many differentially spliced variants, each of which may fulfill subtly different functions. We have studied here two variants that differ at their N-terminal end. The longer variant contains an F3 domain and is expressed in virtually all tissues and at every developmental stage investigated. This isoform seems to represent the major form of collagen XIV. Another variant lacking the N-terminal F3 repeat and starting with a VA domain is primarily expressed in embryonic connective tissues but not in cartilage and bone. It shows a pronounced down-regulation during development and is virtually undetectable after hatching.

Together with other studies, our results demonstrate that chicken collagen XIV contains at least three regions, which are subject to alternative splicing: the first F3 domain at the N terminus (4, 11, this report), a 3-amino acid segment in the center of the molecule (22), and a 31-amino acid segment in the C-terminal NC1 domain (4, 30). Theoretically, alternative splicing of these three regions could give rise to eight different collagen XIV isoforms. Whether all these isoforms are expressed simultaneously or whether there is some way of coordinated regulation remains to be determined.

Collagen XIV is closely related to collagen XII. Collagen XII also occurs in several isoforms (31, 32) that differ at the N terminus (NC3 domain) as well as the C terminus (NC1 domain). At the N terminus, a large region encompassing eight F3 repeats and two VA domains is subject to alternative splicing, whereas at the C terminus, a long NC1 domain with 74 amino acids or a short NC1 domain with 19 amino acids is expressed. In this case, coordinated regulation of alternative splicing has been suggested based on the observation that the expression pattern of the longer NC1 variant is very similar to that of the longer NC3 variant (31). To perform these studies, the authors prepared full-length cDNA molecules for collagen XII with the help of specific primers for the two NC1 isoforms. Analogous experiments with collagen XIV have not been successful. We have not been able to prepare full-length cDNA transcripts for collagen XIV that extended all the way from the region of the NC1 domain to that of the first F3 domain (4). In fact, our results suggested that the structure of the mRNA at the region coding for the first F3 domain must be unusual, because it could not be cloned from commercial cDNA libraries but required cloning from a special primer extension library (4).

What is the physiological role of the different collagen XIV isoforms? Because collagen XIV seems to interact with interstitial collagen fibrils via its COL1 and NC1 domains, alternative splicing within the NC1 domain may affect this interaction. The longer NC1 isoform contains an uncharged region inserted into the otherwise highly charged NC1 domain (4, 30). It was therefore speculated that the longer NC1 variant may favor a strong interaction with the surface of the collagen fibrils and their tapered ends, whereas the shorter isoform may have a lower affinity (30). The function of the two isoforms containing or lacking the three-amino acid insert is less clear. Computer modeling studies suggested that the extra amino acids cause an increase in a flexible loop connecting two β-strands within the sixth F3 domain (22). This increase might affect the arrangement of the domain in the collagen XIV molecule, thereby modulating its interactions with other matrix molecules.

More is known about the putative function of the isoforms containing or lacking the first F3 domain. We as well as another research group (17) could demonstrate that this F3 domain possesses cell binding properties. The isoform without N-terminal F3 domain will therefore lack the capability of interacting with cells. Cell binding to collagen XIV has been observed with hematopoietic (21) as well as fibroblastic cells (17, 20), but not with smooth muscle cells (this report). The interaction is inhibited by heparin at concentrations as low as 0.03 μg/ml. It is also inhibited by other glycosaminoglycans but considerably higher concentrations (1000 μg/ml for heparan sulfate, 30 μg/ml for decorin) are required to obtain half-maximal inhibition (17, 20, this report). Heparin does not only inhibit cell binding, but also interacts directly with the F3 domain and requires 500 mM NaCl for displacement. A second heparin-binding site has been identified by Font et al. (18) at the opposite end of the collagen XIV molecule in the NC1 domain. This site has been analyzed in great detail. It adopts an α-helical fold with a twisted basic groove (16). It shows considerably lower affinity for heparin than the first site, because only 250 mM NaCl is required for displacement of the complex. This observation is in agreement with our studies using full-length collagen XIV polypeptides translated in vitro. The polypeptide containing the N-terminal F3 domain binds to heparin-Sepharose and requires 500 mM salt for displacement. The polypeptide without N-terminal F3 domain also interacts...
with heparin-Sepharose but requires only 150 mM salt for displacement.

We have evidence that cell binding and interaction with heparin represent two functionally distinct properties shared by the first F3 domain. This notion is supported by site-directed mutagenesis experiments. Cell binding is completely abolished when a single amino acid within the F3 domain (Lys-51) is mutated. In contrast, the same mutation does not affect the interaction of the F3 domain with heparin. Thus, the two activities can be distinguished, although they seem to be exerted by spatially overlapping areas. Molecular modeling of the first F3 domain indicates that the mutated Lys is situated within a positively charged groove that resembles the three-dimensional heparin-binding site proposed by Busby et al. (29).

Ehnis et al. (20) have proposed a chondroitin/dermatan sulfate form of CD44 as a cell surface receptor for collagen XIV. This membrane-bound proteoglycan was identified by affinity chromatography of cell surface proteins on collagen XIV immobilized to Sepharose. Based on our results, it does not seem likely that a chondroitin/dermatan sulfate form of CD44 represents the major receptor for collagen XIV. Chondroitin sulfate up to 100 μg/ml did not inhibit binding of cells to the first F3 domain. Furthermore, we were not able to block cell binding to the F3 domain by two different antibodies against human CD44, although both antibodies have been demonstrated to abolish the interaction of CD44 with heparin.2 In addition, U937 and smooth muscle cells that also express CD44 on their surface did not attach to the F3 domain. We therefore favor the idea that the major receptor for collagen XIV is not a chondroitin/dermatan sulfate proteoglycan, but we cannot exclude the possibility that CD44 is involved in cell binding as a coreceptor.

It is possible that the cell binding activity of the first F3 domain is responsible for several biological functions assigned to collagen XIV. Nakagawa et al. (33) identified a chemotactic factor for neutrophils, which turned out to be identical to an N-terminal fragment of collagen XIV. This fragment attracted neutrophils in a concentration-dependent manner, suggesting a role for collagen XIV in neutrophil recruitment and inflammation. Akutsu et al. (34) demonstrated a role of the NC3 domain from collagen XIV in cell migration. When added to fibroblasts cultivated on a reconstituted collagen gel, the isolated NC3 domain inhibited the migration of these cells into the gel. In light of our results, it is conceivable that attraction of neutrophils and inhibition of cell migration are caused by the interaction of the first F3 domain from collagen XIV with its cell surface receptor.

All our results have been obtained with collagen XIV from chick. Human collagen XIV exhibits a highly homologous structure, suggesting that some of the results may also hold true for human collagen XIV. Two isoforms with different NC1 domains have in fact been described for human collagen XIV, although their amino acid sequences and their way of splicing differ considerably from those of the chicken isoforms (12). Alternative splicing of the three-amino acid insert in the sixth F3 domain of collagen XIV has not yet been found in the human protein (12). Likewise, all cDNA clones described for human collagen XIV correspond to the longer isoform containing the F3 domain at the N terminus. In chicken tissues, however, the shorter isoform lacking the N-terminal F3 domain is expressed exclusively before hatching. It therefore remains to be demonstrated whether a similar isoform without cell binding properties may be expressed in human tissues at an early developmental stage.

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