The Type XIII Collagen Ectodomain Is a 150-nm Rod and Capable of Binding to Fibronectin, Nidogen-2, Perlecan, and Heparin*

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Type XIII collagen consists of a short N-terminal intracellular domain, a transmembrane domain, and a collagenous ectodomain, and it is found at many sites of cell adhesion. We report on the characterization of recombinant type XIII collagen. The shed ectodomain was purified from insect cell culture medium and shown to form 240-kDa trimers with a Tm of 42 °C. Correct chain association into a triple-helical conformation was confirmed by limited pepsin digestion and CD spectroscopy. Rotary shadowing electron microscopy of the ectodomain revealed it to be a 150-nm rod with two flexible hinges separating 31-, 52-, and 68-nm portions. The rods represent the collagenous domains 1–3, and the hinges coincide with the non-collagenous domains 2 and 3. By using surface plasmon resonance analysis, the ectodomain showed interaction with immobilized fibronectin, nidogen-2, and perlecan with K0 values in the nanomolar range. The binding sites of type XIII collagen for fibronectin were localized to the collagenous domains, whereas the binding activities for nidogen-2 and perlecan resided in the pepsin-sensitive portions of the ectodomain. Furthermore, the ectodomain bound significantly to heparin, which also inhibited shedding of the ectodomain in insect cell cultures. The results reveal that type XIII collagen is notably distinct in its structure compared with other cell-surface proteins, and the in vitro binding with fibronectin, heparin, and two basement membrane components is indicative of multiple cell-matrix interactions in which this ubiquitously expressed protein participates.

Two of the 19 collagens described in vertebrates belong to the subgroup of transmembrane collagens, namely type XIII and the homedemosomal component, type XVII collagen (1–3). These two nonfibrillar collagens are not structurally homologous except that both have a transmembrane domain. The collagen superfamily also includes other transmembrane proteins that have short collagenous domains, namely the types I and II macrophage scavenger receptors; C1q, the first component of complement C1; a macrophage receptor with a collagenous structure; and the ectodysplasin-A family of proteins (1, 3, 4).

Type XIII collagen molecules reside in the plasma membrane of cells in a type II orientation with a short N-terminal cytosolic domain, a single transmembrane domain, and a large, mainly collagenous ectodomain (5, 6). The N-terminal non-collagenous domain, NC1, of the human protein encompasses a 38-residue cytosolic domain, a 23-residue transmembrane domain, and the first 60 residues of the non-collagenous extracellular sequences adjacent to the plasma membrane (5). The rest of the ectodomain contains three collagenous sequences, COL1–3, with sizes of 104, 172, and 235 residues, respectively, and non-collagenous domains, NC2–4, with sizes of 34, 22, and 18 residues (5). The precursor RNAs encoding human and mouse type XIII collagen are known to be subject to complex alternative splicing, and hence the sizes of the COL1, NC2, COL3, and NC4 domains in humans can vary in the ranges 57–104, 12–34, 184–235, and 13–18 residues, respectively (7–10). Type XIII collagen produced in insect cells forms homotrimers, and the three collagenous domains fold into a stable triple-helical conformation (6). The purified protein has been shown to interact with α1β1 integrin, which is known to bind various collagenous (11).

Type XIII collagen has a wide tissue distribution and has been localized to many sites of cell-matrix interaction in tissues and organs such as the myotendinous junctions in skeletal muscle and at some cell-cell interaction sites such as the intercalated disc of heart muscle (12–15). Type XIII collagen has been localized to focal adhesions in cultured fibroblasts (14). These findings suggest that it is involved in cellular adhesion and that it could interact with matrix components. This present study was initiated to gain information on the molecular dimensions and chemical properties of the ectodomain of this collagen type and to explore the possible interactions of this molecule with a range of matrix components.

EXPERIMENTAL PROCEDURES

Sources of Proteins and Antibodies—Human fibronectin was obtained from Roche Molecular Biochemicals. Human vitronectin was prepared from plasma (16). Human BM-40 (17), mouse nidogen-1 (18), and human nidogen-2 (19) were obtained in recombinant forms from EBNA-293 cells. A mouse laminin-1-nidogen-1 complex and mouse perlecan were prepared from the mouse Engelbreth-Holm-Swarm tumor (20). The microfibrillar components fibulin-1 (21) and fibulin-2 (22) were prepared by recombinant production. Human collagens IV (23) and VI (24) were isolated from placental pepsin digests and consisted mainly of the triple-helical domains. All proteins used for binding analysis were more than 95% pure, as judged by SDS-PAGE, and showed binding activities in other experiments (25). Human types I and III collagens were obtained from Collaborative Biomedical Products

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1 The abbreviations used are: NC1–4, non-collagenous domain 1–4; COL1–3, collagenous domain 1–3; BSA, bovine serum albumin; TBS, Tris-buffed saline; ELISA, enzyme-linked immunosorbent assay.
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(Bedford, MA). The antibodies used in this study were antiserum against the NC3 domain (5), antiserum against the NC2 domain (6), and antiserum against the NC4 domain of type XIII collagen (6). A monoclonal antibody against the recombinant human type XIII collagen ectodomain was produced and pre-screened with an enzyme-linked immunoassay using an ELISA column (ELISA, VTT Biotechnology, Espoo, Finland). The positive sera of the immunized mice were further tested with Western blotting (data not shown). One mouse was chosen for final boosting, and only one of the culture media of the fused cells showed positive signals in ELISA and Western blotting. The fusion was cloned further, and the IgG of one clone was affinity-purified.

Purification of the Recombinant Collagen Ectodomain and Pepsin Fragments—For isolation of the type XIII collagen del1–38 ectodomain, the previous purification protocol (11) was modified. A suspension culture of High Five insect cells (1 × 10⁶ cells/ml, Invitrogen) was coinfected in serum-free Express Five medium (Invitrogen) with del1–38 virus encoding human type XIII collagen (6) (multiplicity of infection = 5) and with the virus 4PH, encoding both the α and β subunits of human prolyl 4-hydroxylase (26) (multiplicity of infection = 1). Forty eight hours post-infection, cell culture medium (400 ml) was separated from the cells by centrifuging at 340 × g for 10 min and filtered through a 0.22-μm membrane and applied to a HiTrap Q 5-ml column (Amersham Biosciences AB). The unbound portion that contained the type XIII collagen was eluted with 100 mM acetate, pH 4.0, and the α chains lacking the cytosolic domain (6) (multiplicity of infection = 5) and with the virus 4PH, encoding both the α and β subunits of human prolyl 4-hydroxylase (26) (multiplicity of infection = 1). Forty eight hours post-infection, cell culture medium (400 ml) was separated from the cells by centrifuging at 340 × g for 10 min and filtered through a 0.22-μm membrane and applied to a HiTrap Q 5-ml column (Amersham Biosciences AB) and eluted using a gradient protocol on an AKTA explorer 10 (Amersham Biosciences AB). The fractions that contained the type XIII collagen ectodomain were concentrated to 1 ml and purified on a Sephacryl S-500 column (1.6 × 100 cm, Amersham Biosciences AB) using 20 mM HEPES, pH 7.0, 0.15 M NaCl. The purity of the elution fraction was analyzed by native gel electrophoresis (27) and SDS-PAGE combined with Coomassie Blue staining and immunoblotting. The purified sample was adapted to 50 mM acetic acid using an Ultrafree Centrifugal Filter Device (molecular mass cut-off 100 KDa, Millipore, Bedford, MA) and then lyophilized.

For pepsin digestion, the purified type XIII collagen ectodomain was dissolved in 70 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and the pH was adjusted to 2 with 0.3 M HCl. The protein was then digested with pepsin (100:1 w/w) for 30–60 min at 22 °C. The reaction was stopped by neutralization to pH 7.0. For isolation of pepsin-resistant fragments, the digested mixture was applied to a Superdex 2000 gel filtration column (1.6 × 60 cm, Amersham Biosciences AB) and subsequently eluted with a 50 mM sodium phosphate buffer, pH 7. Dis, containing 0.15 M NaCl. The pepsin-digested fragments were purified with AKTA Explorer 10 (Amersham Biosciences AB). All the fractions were analyzed by SDS-PAGE and detected with Coomassie Blue staining and Western blotting.

Rotary Shadowing Electron Microscopy—Glycerol spraying and rotary shadowing for electron microscopy were performed as described previously (28). Briefly, the samples were diluted to a final concentration of 50 μg/ml with appropriate volumes of 40% glycerol, sprayed onto microcones, and dried in a vacuum, and rotary shadowed with platinum/carbon.

Protein Analysis—The amount of purified type XIII collagen ectodomain was determined by a Micro BCA assay according to the supplier’s instructions (29). Amino acid analysis was performed in a 421 amino acid analyzer (Applied Biosystems, Foster City, CA) using a manual gas phase hydrolysis method according to the manufacturer’s protocol. The N-terminal protein sequence were determined with a 477A protein sequencer (Applied Biosystems) with samples electroblotted onto the ProBlott membrane (Applied Biosystems) (30).

CD spectroscopy was carried out at 15 °C using a Jasco J710 spectropolarimeter (Jasco Inc., Easton, MD). The far-UV spectra of proteins were measured from 195 to 250 nm in 50 mM acetic acid. The melting temperature was obtained by heating the sample at 1 °C/min until the samples were dissolved in 70 mM Tris-HCl, 300 mM NaCl, 0.2% Triton X-100, pH 7.5, containing Complete™ protease inhibitor mixture (Roche Molecular Biochemicals) at 0 °C for 30 min, and centrifuged at 17,000 × g for 30 min to remove the insoluble material. The supernatant was loaded at 1 ml/min onto a HiTrap heparin column (Amersham Biosciences AB), which was pre-equilibrated with the same homogenization buffer at 4 °C. The column was eluted in steps with the same buffer containing 0.5 and 1 mM NaCl.

RESULTS

Purification and Characterization of Recombinant Type XIII Collagen—We have previously (6, 11) generated recombinant baculoviruses that direct the synthesis of full-length (virus whumanXIII) and N-terminally truncated type XIII collagen chains (del1–38) lacking the intracellular portion (Fig. 1). Both the full-length and the N-terminally deleted chains are known to form triple-helical molecules, but the del1–38 protein is expressed in higher amounts and shed significantly more efficiently from insect cells than the full-length type XIII collagen (32). In order to study the chemical, structural, and binding properties of type XIII collagen, we have now isolated it from the insect medium of High Five insect cells coinfected with the del1–38 virus and a virus 4PHβα encoding the enzyme prolyl 4-hydroxylase, which is needed to obtain sufficient hydroxylation of proline residues in the recombinant collagen chains (33, 34). The purification protocol described previously (11) was modified (see “Experimental Procedures”), resulting in improved purity and a yield of 0.5–1 mg of purified protein per liter of culture medium. The purity of the recombinant protein was pelleted 48 h post-infection, homogenized with 50 ml of 70 mM Tris-HCl, 300 mM NaCl, 0.2% Triton X-100, pH 7.5, containing Complete™ protease inhibitor mixture (Roche Molecular Biochemicals) at 0 °C for 30 min, and centrifuged at 17,000 × g for 30 min to remove the insoluble material. The supernatant was loaded at 1 ml/min onto a HiTrap heparin column (Amersham Biosciences AB), which was pre-equilibrated with the same homogenization buffer at 4 °C. The column was eluted in steps with the same buffer containing 0.5 and 1 mM NaCl.

Binding Assays—Surface plasmon resonance assays were performed with a BIAcore® 3000 instrument (Biacore AB, Uppsala, Sweden). Proteins were immobilized on CM5 sensor chips (research grade) (Biacore AB) with the standard amine coupling chemistry (25). They were immobilized at different levels from 200 to 2000 resonance units for affinity analyses or binding kinetics studies. Binding analyses were carried out in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl containing 0.05% P-20 surfactant (Biacore AB) at a flow rate of 20 μl/min. The association phase was monitored for 3 min, and the dissociation curve was recorded for 10 min. The bulk effects were subtracted using reference surfaces. The chips were regeneration by treatment with 2.5 ml of NaCl for 2 min. In the case of regenerating a BM-40 immobilized chip, 10 s injections of 2.5 mM NaCl containing 5 mM NaOH were applied three times. For the calculation of kinetics constants, the association and dissociation phases of the sensorsgrams for each protein analyte at different concentrations (0–1000 nM) were fitted globally to the available models with BIAevaluation software (Biacore AB) for rate constants calculation were chosen based on the residual plots, the parameters of X², and visual inspections of the fitting curves.

Solid phase assays were performed following a published protocol (31) with some modifications. Various proteins (5 μg/ml) were coated onto the plastic surface of microtiter wells at 4 °C overnight. All the other steps were performed at room temperature. The wells were blocked with 1% BSA (crystallized, SEBVA, Heidelberg, Germany) in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl (TBS) for 1.5 h and then washed with TBS, 0.05% Tween 20, and incubated for 2.5 h with type XIII collagen ectodomain as a soluble ligand serially diluted in 1% BSA, 2 mM CaCl₂, and TBS. After thorough washing, the wells were incubated with an antibody against the NC3 domain of type XIII collagen (33) (del1–38) diluted in 1% BSA, 2 mM CaCl₂, and TBS, followed by a monoclonal antibody against the secondary antibody conjugated with horseradish peroxidase. The amount of bound ligand was detected by adding 5-aminosalicylic acid (Sigma) in the presence of 0.01% H₂O₂. The detection was performed at 490 nm.

For the heparin binding studies the microtiter wells were coated with 10 μl/g of heparin-BSA (Sigma), and the type XIII collagen ectodomain added as a soluble ligand. A monoclonal antibody against the type XIII collagen ectodomain was applied as the first antibody, and the secondary antibody was anti-mouse IgG conjugated with horseradish peroxidase. 3,3′,5,5′-Tetramethylbenzidine (Sigma) was used as the substrate, and detection took place at 450 nm. The heparin binding was further confirmed by means of a HiTrap heparin column (5 ml, Amersham Biosciences AB). The purified type XIII collagen protein was applied to a 10 mM homogenization buffer containing 0.15 M NaCl, equilibrated HiTrap heparin column at a flow rate of 0.1 ml/min and then eluted at 1 ml/min using a linear gradient of NaCl.
was more than 95% as analyzed by SDS-PAGE under reducing and non-reducing conditions and by acidic native gel electrophoresis (data not shown). Non-reduced SDS-PAGE revealed a single band with molecular mass of about 240 kDa, whereas the reduced sample had an apparent molecular mass of 70–80 kDa, indicating that the isolated protein is a disulfide-bonded trimer. The presence of a single band in the acidic native gel electrophoresis confirmed the homogeneity of the purified protein.

The purified collagen XIII was subject to amino acid analysis (Table I). A close match was observed between the measured amino acid composition and the predicted data based on the cDNA sequence, indicating that the purified protein did not contain any significant impurities. Because proline or lysine residues in the Lys position of XYG amino acid triplets in collagens are subject to hydroxylation (35), the amino acid sequence of the del1–38 ectodomain would imply that 71.7% of the available prolines and 30% of the available lysines were hydroxylated (Table I).

Identification of Three Collagenous Pepsin-resistant Domains—Pepsin digestion of crude insect cell extract had shown previously (6) pepsin-resistant fragments of 19–29 kDa detectable by Western blotting and containing the COL1 domain, 30- and 32-kDa fragments containing the COL2 domain, and a 36-kDa fragment containing the COL3 domain. In the present case, limited pepsin digestion of the purified type XIII collagen ectodomain revealed four pepsin-resistant fragments of 20, 29, 34, and 39 kDa (Fig. 2). These were subjected to N-terminal protein sequence analysis, which showed that the cleavages occurred in the non-collagenous domains NC1, NC2, and NC3 (see Figs. 1 and 2). The sequenced fragments included four prolines in positions amenable to hydroxylation, and each one of these was indeed found hydroxylated (see Fig. 2). The 20-kDa band represented the COL1 domain and 13 residues derived from NC1, whereas the 29-kDa band represented the COL2 domain, including 10 extreme C-terminal residues of the NC2. The two bands with molecular masses of 34 and 39 kDa represented the COL3 domain, and both included the four extreme C-terminal residues of the 21-residue NC3. Antibody XIII/NC4–SO against the extreme C-terminal end of type XIII collagen (6) recognized both the 34- and 39-kDa bands (data not shown).

The 34- and 39-kDa pepsin fragments were subsequently separated by gel filtration and their amino acid compositions analyzed. The only difference between the two fragments was a low level of hydroxylation of proline and lysine residues in the former compared with the latter (data not shown), indicating some heterogeneity produced in the protein. Low hydroxylysine levels may lead to decreased glycosylation and thus could account for the difference in size between the 34- and 39-kDa COL3 domain fragments.

Type XIII collagen contains several cysteine residues, which can form either intrachain or interchain disulfide bonds (see Fig. 1). When the purified pepsin-resistant fragments were analyzed by SDS-PAGE under non-reducing conditions, the mobility of the COL2- and COL3-derived fragments were the same as detected in reduced SDS-PAGE, whereas the COL1 fragment had a reduced mobility, with an apparent molecular mass of 46 kDa (data not shown).

Molecular Shape and Conformation of the Type XIII Collagen Ectodomain—The size and shape of type XIII collagen can be predicted from the sizes of fibril-forming collagen molecules, whose uninterrupted collagenous domains of about 1000 amino acid residues have lengths of around 300 nm (36). Depending on the splice variant in question, the ectodomain of human type XIII collagen is 581–706 residues in length (8) and thus can be predicted to be composed of three rod-like domains with sizes of about 30, 50, and 70 nm and probably flexible hinges between them, formed by the short non-collagenous domains NC2 and NC3. Electron microscopy after rotary shadowing of the purified ectodomain revealed rod-like molecules typical of triple-
helical collagens (Fig. 3). The predominant molecule population had a length of about 150 nm (Fig. 3), and many had one or two kinks in them, which are likely to coincide with the NC2 and NC3 domains (see Fig. 1). In the former case the dimensions were estimated to be $30.3 \pm 1.1$ and $125.8 \pm 3.7$ nm, and in the latter case $31.2 \pm 1.3, 52.8 \pm 2.7$, and $68 \pm 4.9$ nm. In each case five molecules were measured. In relation to the known primary structure, it appears that COL1 is $31$ nm in length, COL2 $53$ nm, and COL3 $68$ nm.

**Thermal Stability of the Ectodomain**—We have assayed previously the thermal stabilities ($T_m$) of insect cell-produced type XIII collagen using a combination of pepsin and trypsin-chymotrypsin digestions of cell extracts and detection of the resistant fragments by Western blotting (6). According to this analysis the $T_m$ values of the COL1, COL2, and COL3 domains were $38, 49$, and $40^\circ C$, respectively. Here the secondary protein structure of the purified type XIII collagen ectodomain was studied using CD spectroscopy, which demonstrated a typical collagenous conformation with molar ellipticities ($\theta$ of $-29,000$ at $198$ nm and $+2100$ at $222$ nm (Fig. 4). This ectodomain showed a broad melting profile from $27$ to $42^\circ C$ indicating that some molecules are less stable, presumably due to under-hydroxylation (data not shown). The $T_m$ is critically dependent on the level of proline 4-hydroxylation, and previous studies (26, 33) on the expression of collagen types I–III in insect cells have shown that it is also dependent on the exogenous enzyme. Because the insect cells are infected with two separate viruses, one directing synthesis of type XIII collagen and another synthesis of prolyl 4-hydroxylase, it is possible that not all cells are equally coinfected with both, resulting in under-hydroxylation of a part of the recombinant collagen. Indeed, this was demonstrated by amino acid analysis of the 34- and 39-kDa COL3 domain pepsin fragments (see above), pointing to a variability in the level of hydroxylation of the synthesized recombinant type XIII collagen molecules that is likely to account for the heterogeneity in the melting points. We presume that the highest melting point, $42^\circ C$, represents the $T_m$ of the molecules with the highest level of proline 4-hydroxylation and that the molecules with $T_m$ values lower than body temperature are likely to represent under-hydroxylated cases.

**Binding of the Type XIII Collagen Ectodomain to Extracellular Proteins**—The wide occurrence of type XIII collagen in cell-matrix contact regions, including basement membrane zones (12, 14), prompted us to investigate its binding to widely occurring matrix components and ones concentrated in basement membranes. We tested a series of extracellular matrix proteins using a surface plasmon resonance assay with several matrix proteins as immobilized ligands and the type XIII collagen as a soluble analyte and vice versa. In the former case, type XIII collagen ectodomain could bind to fibronectin (Fig. 5A), nidogen-2 (Fig. 5B), and perlecan (Fig. 5C), and the plateau response level was observed to be dose-dependent after reference subtractions to compensate for the bulk effects. However, the ectodomain failed to interact with immobilized vitronectin, a laminin-1-nidogen-1 complex, nidogen-1, type IV collagen, type VI collagen, fibulin-1, or fibulin-2 (data not shown). Furthermore, type XIII collagen showed high binding affinities to BM-40 resulting in difficulties in dissociation. Repeated injections of $2.5$ mM NaCl solution containing $5$ mM NaOH were needed to regenerate the sensor chip. Because no data were available for the resistance of BM-40 to this regeneration condition, the binding kinetics studies on type XIII collagen to BM-40 were not pursued further.

All the sensorgrams for fibronectin, nidogen-2, and perlecan were evaluated with the fitting models in BLIAevaluation software 3.1, including Langmuir 1:1 binding (A + B ↔ AB), parallel (A + B ↔ AB, AB + B ↔ AB2), competitive (A1 + B ↔ A1B, A2 + B ↔ A2B), and two-state reactions (A + B ↔ AB ↔ AB*), asterisk indicates changed conformation). The models of Langmuir 1:1 with mass transfer and Langmuir 1:1 with baseline drift were not applied because no mass transfer was detected by analyzing $\ln(dR/dt)$ versus time plots, and the baseline drift was eliminated as judged by the appearance of the straight baseline observed obtained with blank injections (37). In the case of the interaction between the type XIII collagen ectodomain and immobilized fibronectin, distinct association and dissociation phases were noted, but none of the fittings to the available models showed a $\chi^2$ value lower than 10, which implied a more complicated reaction between the two molecules. For the interaction to nidogen-2 and perlecan, the conformational change model (a two-state reaction) resulted in $\chi^2$ values of 0.37 for nidogen-2 and 1.8 for perlecan. Because further studies on identifying the multiple binding sites between type XIII collagen and fibronectin, such as association and dissociation phases were noted, but none of the fittings to the available models showed a $\chi^2$ value lower than 10, which implied a more complicated reaction between the two molecules. For the interaction to nidogen-2 and perlecan, the conformational change model (a two-state reaction) resulted in $\chi^2$ values of 0.37 for nidogen-2 and 1.8 for perlecan. Because further studies on identifying the multiple binding sites between type XIII collagen and fibronectin, as well as the conformational changes of the complexes of type XIII collagen-nidogen-2 and type XIII collagen-perlecan are beyond the scope of the present paper, the kinetic constants were analyzed using global fitting of $k_a$ and $k_d$ separately in the Langmuir 1:1 model (Fig. 5). The apparent $K_p$ values were determined from the ratios of $k_a/k_d$ (Table II).

The binding of type XIII collagen to fibronectin, nidogen-2, and perlecan was confirmed by using type XIII collagen as a soluble protein in an ELISA solid phase assay, showing that the concentrations of the ectodomain required for half-maximal binding to fibronectin, nidogen-2, and perlecan could be determined as 2.5, 4.5, and 9 nM, respectively (data not shown).

In the reverse surface plasmon resonance experiment, the
Table II  

Binding affinity of the type XIII collagen ectodomain for fibronectin, nidogen-2, and perlecan in a surface plasmon resonance assay

| Immobilized ligand | Fibronectin | Nidogen-2 | Perlecan |
|--------------------|-------------|-----------|----------|
| $k_a \times 10^6$ (M$^{-1}$ s$^{-1}$) | 1.9 ± 0.3 | 1.4 ± 0.3 | 2.7 ± 0.2 |
| $k_d$ (nM) | 2.4 ± 0.2 | 2.5 ± 0.4 | 3.0 ± 0.5 |
| $x^2$ for $k_a$ fitting | 1.9 ± 0.3 | 2.0 ± 0.5 | 1.9 ± 0.3 |
| $x^2$ for $k_d$ fitting | 4.6 ± 0.4 | 3.7 ± 0.3 | 4.2 ± 0.4 |

Structure and Binding Properties of Type XIII Collagen

Fibronectin, nidogen-2, and perlecan were immobilized on CM5 sensor chips. The type XIII collagen ectodomain was tested at different concentrations, i.e. at 0–25 nM for fibronectin binding and at 0–75 nM for nidogen-2 and perlecan analyses. The kinetic rate constants were analyzed using separate fitting of $k_a$ and $k_d$ in a Langmuir 1.1 binding model (A + B ⇄ AB), and the parameters are means ± S.D. of three independent determinations.

Matrix proteins to the immobilized type XIII collagen ectodomain was also analyzed in the solid phase assays using specific antibodies against each individual soluble analyte. High affinity of fibronectin and nidogen-2 to type XIII collagen was confirmed, and vitronectin, perlecan, nidogen-1, fibulin-2, and types IV and VI collagen showed moderate interaction (data not shown). No significant binding was observed with collagen IV, laminin-1-nidogen-1 complex, or BM-40 (data not shown). Furthermore, the fibrillar collagen types I and III were also tested using both surface plasmon resonance and solid phase assays, and no significant interaction was observed (data not shown).

The interaction between different collagenous domains of type XIII collagen (pepsin-digested fragments) and immobilized fibronectin, nidogen-2, and perlecan, which were previously shown to bind to the soluble ectodomain, was also tested. In this case all the collagenous fragments bound to fibronectin although less strongly compared with the ectodomain, and none of them showed significant interaction to nidogen-2 or perlecan (data not shown).

Binding of Type XIII Collagen to Heparin—While developing purification methods for type XIII collagen, we found that the del1–38 protein binds to a heparin column with high affinity. High Five cells were coinfected with the viruses del1–38 and 4PHαβ; cell and medium fractions were collected 48 h post-infection; the cells were homogenized in a buffer containing protease inhibitors, and the ensuing supernatant was loaded onto a heparin column. The cellular type XIII collagen was tightly bound to the heparin, as 1 M NaCl was needed for its elution (data not shown). The eluted del1–38 type XIII collagen protein was aggregated to an insoluble form when the concentration of the salt in the sample buffer was lowered, rendering purification and further work with the del1–38 protein difficult. This is likely to be due to the fact that the cellular del1–38 protein contains the hydrophobic transmembrane domain. In a solid phase assay binding test, the type XIII collagen ectodomain was found to bind to immobilized heparin-BSA with half-maximal saturation at 1.8 nM, whereas BSA itself did not show any binding (Fig. 6A). The binding of the type XIII collagen ectodomain to heparin was further confirmed by heparin affinity chromatography, in which the ectodomain was eluted with 0.7 M NaCl (Fig. 6B).

Heparin Inhibits Shedding of Type XIII Collagen—We have shown previously that the recombinant type XIII collagen synthesized by insect cells could be released into the medium by ectodomain cleavage and that this cleavage probably involves a furin-type protease (32). Because heparin-like proteoglycans occur abundantly in the matrix and pericellular environment, we tested the possibility of heparin inhibiting the shedding of type XIII collagen into the culture medium. High Five cells were cultured in a serum-free medium containing 57 μg/ml heparin for 48 h, coinfected with viruses del1–38 and 4PHαβ,
using a linear gradient of NaCl. Type XIII collagen was eluted with 0.7 M heparin column at a flow rate of 0.1 ml/min and then eluted at 1 ml/min was applied to a 10 m M HEPES, 0.15 M NaCl-equilibrated HiTrap main were tested by a solid phase assay using a monoclonal antibody plates, and different concentrations of the type XIII collagen ectodomain revealed a 150-nm long rod with two flexible hinges and all three triple helical domains being well suited for various protein corresponds to nearly all of the ectodomain, including the NC3 domain. The sequencing data suggest that the purified shedding ectodomain, Lys-247 in the NC2 domain, and Leu-469 in the NC1 domain (coinciding with the N-terminal end of the ectodomain). The elongated structure of the ectodomain revealed three collagenous domains, and N-terminal dissociation were evaluated in the phases of good fits, monovalent molecules (37). Therefore, the 1:1 Langmuir model most extracellular matrix proteins consist of two or more subunits. However, the kinetic constant analysis using Biacore results in only 10% of the type XIII collagen being found in the medium, as opposed to 50% in the absence of heparin (Fig. 7, B and C). There is no significant difference of type XIII collagen expression level in the cells with or without heparin (Fig. 7A), indicating that the heparin treatment did not have any effect on the biosynthesis of type XIII collagen. Thus heparin may be said to inhibit shedding of type XIII collagen into the culture medium.

DISCUSSION

This paper reports on the first characterization of purified type XIII collagen. Insect cells were used to express the human collagen together with the α and β subunits of human prolyl 4-hydroxylase; the latter is known to be necessary for the generation of correctly hydroxylated recombinant collagen molecules (33). Purification of membrane-bound recombinant type XIII collagen was difficult due to aggregation, and thus a purification method was developed for the ectodomain portion, which is known to be shed via a furin protease cleavage (6). The shed ectodomain is a trimer consisting of three identical 560-residue α1(XIII) chains (residues 109–668) of the full-length α chain without any sequences corresponding to alternatively spliced exons 13, 29, and 34, and was isolated from the infected insect cell medium by anion exchange, cation exchange, and gel filtration chromatographies. Pepsin digestion of the purified ectodomain revealed three collagenous domains, and N-terminal sequencing identified the cleavage sites at residues Glu-109 in the NC1 domain (coinciding with the N-terminal end of the shed ectodomain), Lys-247 in the NC2 domain, and Leu-469 in the NC3 domain. The sequencing data suggest that the purified protein corresponds to nearly all of the ectodomain, including all three triple helical domains being well suited for various structural and functional studies.

Rotary shadowing electron microscopy of the purified ectodomain revealed a 150-nm long rod with two flexible hinges separating 31-, 52-, and 68-nm segments (Fig. 8A). Based on the cDNA-derived primary structure, it is apparent that the rods represent the COL1 (95 residues in length), COL2 (172 residues), and COL3 (209 residues) domains, with flexible regions coinciding with the NC2 (31 residues) and the NC3 (22 residues) domains. The elongated structure of the ectodomain of type XIII collagen could potentially extend a considerable distance from the cell surface, and it also may bend toward the plasma membrane (Fig. 8B). This collagen type is notably distinct in its structure from the other cell surface proteins with which it codistributes in cells and tissues, such as the integrins, whose ectodomain is about 20 nm in length (38, 39).

FIG. 6. Binding of the type XIII collagen ectodomain to heparin. A, BSA (●) and heparin-BSA (■), were immobilized on microtiter plates, and different concentrations of the type XIII collagen ectodomain were tested by a solid phase assay using a monoclonal antibody against the ectodomain. B, the purified type XIII collagen ectodomain was applied to a 10 m M HEPES, 0.15 M NaCl-equilibrated HiTrap heparin column at a flow rate of 0.1 ml/min and then eluted at 1 ml/min using a linear gradient of NaCl. Type XIII collagen was eluted with 0.7 M NaCl.

The hydroxylation of proline and lysine residues in type XIII collagen in tissues has not been determined as yet. Coexpression of the prolyl 4-hydroxylase in insect cells with the fibrillar collagens I–III has resulted in 4-hydroxyproline levels that are comparable with those of native collagens (26, 33). With respect to the type XIII collagen ectodomain produced in insect cells, 72% of the prolines in positions amenable to hydroxylation (77/1000 amino acid residues) and 30% of such lysines (17/1000 residues) were found to be modified. These modification levels resemble those reported for other collagens (35). Other modifications identified were the presence of an interchain disulfide bridge in conjunction with the COL1 domain. Because both ends of this domain are flanked with cysteine residues, the disulfide bridge may be located at one or both ends. The C-terminal end of the α1(XIII) chain contains two cysteine residues, but these did not form interchain bonds and are thus presumed to form one intrachain disulfide bond. Enzymatic digestions of insect cell extracts combined with Western blotting detection of the digestion products have revealed previously (6) Tm values of 38, 49, and 40 °C for the COL1, COL2, and COL3 domains, respectively. The present CD spectrum analysis suggests that the purified ectodomain has a Tm of 42 °C.

The binding capacities of the purified recombinant ectodomain were tested against a number of matrix molecules using a surface plasmon resonance assay and ELISA solid phase assay. A high affinity for fibronectin was detected, with a KD of 2.4 nM when using type XIII collagen as the soluble analyte and a KD of 17.5 nM when using fibronectin as the soluble analyte. It should be noted that studies on some other matrix molecules have also shown Kd values which somewhat differ depending on whether the molecules are used as immobilized ligands or soluble analytes (40). The binding reaction between type XIII collagen and fibronectin as well as nidogen-2 and perlecangen was complex because type XIII collagen is a trimeric molecule, and most extracellular matrix proteins consist of two or more subunits. However, the kinetic constant analysis using Biacore evaluation software is mostly suitable for monomeric and monovalent molecules (37). Therefore, the 1:1 Langmuir model was used for curve fitting, and the rate constants for association and dissociation were evaluated in the phases of good fits, and all the calculated data were considered biologically and experimentally relevant.

Fibronectin is a prominent constituent of the extracellular matrix around many cells and is known to play important roles in cell adhesion and migration during development and wound healing and also to affect many cellular functions, including
The extensive occurrence of type XIII collagen in tissues includes most cell-basement membrane interfaces (14, 15). It was thus of interest to test its binding to various basement membrane components. These included nidogen, existing in two isoforms, nidogen-1 and nidogen-2 (19), and perlecan, a multifaceted modular heparan sulfate proteoglycan (42). Type XIII collagen was found to bind to the immobilized nidogen-2 with high affinity, the $K_D$ being about 5.4 nM. Furthermore, type XIII collagen bound to immobilized perlecan with a $K_D$ of 2.5 nM. Nidogen-2 and perlecan did not show considerable interactions in the surface plasmon resonance assays when type XIII collagen ectodomain was immobilized, indicating that the immobilization on the sensor chip resulted in masking of some of the binding sites. Another major basement membrane protein, BM-40, showed tight binding to type XIII collagen in the surface plasmon resonance assay, but no apparent interactions were observed in the ELISA solid phase assay. This result suggested that the association between BM-40 and type XIII collagen might not be biologically specific. Altogether, using type XIII collagen ectodomain as the immobilized ligand, most molecules tested, possibly due to low binding or nonspecific aggregation of the matrix proteins tested. Moreover, testing the binding properties of the purified pepsin-resistant fragments of type XIII collagen, namely fragments representing the COL1, COL2, and COL3 domains, revealed only very limited binding to these collagenous domains. More specifically, the three collagenous domains bound only to immobilized fibronectin. These data suggested that most of the binding affinities of the type XIII collagen ectodomain for nidogen-2 and perlecan reside in its pepsin-sensitive portions.

Interestingly, the type XIII collagen ectodomain also showed positive binding to heparin. The apparent affinity was close to another heparin-binding protein, the basic fibroblast growth factor-2, which revealed half-maximal saturation at 2 nM as analyzed with the same method (43). High heparin affinity suggests a potential to bind matrix or membrane-bound heparan sulfate proteoglycans through clusters of basic amino acids that are abundantly existing in the type XIII collagen molecule (44). Because heparin-related proteoglycans occur both as cellular receptors and in the fibrillar matrix and basement membranes, this observation may further enlarge the list of potential matrix molecules binding to type XIII collagen to include interactions with proteoglycan receptors. Furthermore, heparin inhibited the shedding of the type XIII collagen ectodomain into the insect cell culture medium. The mechanism for this inhibition is not clear. However, it has been demonstrated that clustered arginines are essential for heparin binding (45), and possibly heparin masks the arginine-rich furin cleavage site of type XIII collagen (32). All in all, the data may suggest a role for heparin-related molecules in regulating cleavage of the type XIII collagen in tissues.

The basement membrane components form a complicated network in which the various members interact with each other and with cellular receptors. Type IV collagen is known to bind to nidogen and the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (19, 45, 46). Perle-
can is cell-adhesive through β1 integrins and can bind to heparin and several extracellular matrix proteins such as nitro-
gen-1 and fibulin-2 (47). In light of the location of type XIII collagen along basement membranes and the in vivo binding of the ectodomain with nidogen and perlecan, and possibly with types IV and VI collagen and fibulin-2, it is likely that this collagen serves to mediate the adhesion of cells to the basement membranes. It is also possible that a portion of the type XIII collagen molecules may be shed into the matrix and may serve structural or regulatory functions. Mutant mice synthesizing type XIII collagen molecules lacking the cytosolic and trans-
meric strength in cell-matrix and some cell-cell adher-
tially very deep penetration into the matrix of the largely
with other collagens and matrix molecules (36). With its poten-
touristic matrix proteins such as nido-
progressive muscular disorder, as they demonstrated abnor-
type XIII collagen molecules lacking the cytosolic and trans-
and perlecan, and possibly also some other basement mem-
and several extracellular matrix proteins such as nido-
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The Type XIII Collagen Ectodomain Is a 150-nm Rod and Capable of Binding to Fibronectin, Nidogen-2, Perlecain, and Heparin

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