Ryudocan, a heparan sulfate proteoglycan, was isolated from human endothelium-like EAhy926 cells by a combination of ion-exchange and immunoaffinity chromatography. Purified human ryudocan has biochemical properties similar to those of rat ryudocan isolated from microvascular endothelial cells. Human ryudocan contains only heparan sulfate (HS) glycosaminoglycan chains along with a core protein with an apparent molecular mass of 30 kDa. We evaluated the interactions between purified human ryudocan and several extracellular ligands by using a solid-phase binding assay. We found that basic fibroblast growth factor (bFGF), midkine (MK), and tissue factor pathway inhibitor (TFPI) exhibit significant ryudocan binding. Heparinase (but not chondroitin ABC lyase) treatment destroyed the ability of ryudocan binding to bFGF, MK, and TFPI. Heparin and HS, but not chondroitin sulfate, inhibited such ryudocan binding. Thus, the HS chains of ryudocan appear to be responsible for its binding to bFGF, MK, and TFPI. The apparent dissociation constants for purified ryudocan were as follows: bFGF, 0.50 nM; MK, 0.30 nM; and TFPI, 0.74 nM. Immunohistochemical analysis revealed that ryudocan was expressed in fibrous connective tissues, peripheral nerve tissues, and placental trophoblasts. These findings suggest that ryudocan may possess multiple biological functions, such as bFGF modulation, neurite growth promotion, and anticoagulation, via HS-binding effectors in the cellular microenvironment.

Ryudocan is an integral membrane heparan sulfate proteoglycan (HSPG) that was originally isolated from endothelial cells as an anticoagulant molecule (1, 2). This compound is a member of the syndecan family composed of four cell membrane intercalated HSPGs: syndecan (syndecan-1), fibroglycan (syndecan-2), N-syndecan (syndecan-3), and ryudocan (syndecan-4), which have homologous transmembrane and intracellular domains, but very distinct extracellular regions (3). The best characterized molecule of this family is syndecan, the prototypical member that was isolated from mouse mammary epithelial cells (4). Syndecan selectively binds via its heparan sulfate (HS) chains to a variety of matrix components, including fibrillar collagens (5), fibronectin (6), thrombospondin (7), tenasin (8), and amphoterin (9), which suggests that syndecan may be an extracellular matrix receptor.

Syndecan also binds to the heparin-binding growth factors, such as basic fibroblast growth factor (bFGF) (10–14). The potential importance of this interaction is underscored by recent reports demonstrating the involvement of cell-surface HSPGs in bFGF signaling mechanisms (15). N-Syndecan is another member of the syndecan family. This molecule was recently identified and cloned in rat Schwann cells and chicken embryos (16, 17). N-Syndecan possesses a high degree of specificity for bFGF through its glycosaminoglycan (GAG) chains (18). N-Syndecan was also isolated as a heparin binding growth-associated molecule (pleiotrophin) receptor and was anticipated to mediate the neurite outgrowth-promoting signal from a heparin binding growth-associated molecule to the cytoskeleton of growing neurites (19).

Several syndecan-4 cDNAs have been characterized for the rat (ryudocan (2)), human (ryudocan (20) and amphiglycan (21)), and chicken (22) forms. Little is known, however, about the biological function of this molecule. Thus, we purified human ryudocan from endothelium-like cells (EAhy926 cells) and examined its interactions with various biological ligands by using a solid-phase binding assay. The ligands tested for ryudocan binding were bFGF, midkine (MK), and tissue factor pathway inhibitor (TFPI). Among them, MK is a heparin-binding growth factor unrelated to fibroblast growth factor (23, 24), promotes neurite outgrowth (25) and neuronal cell survival (26), and enhances plasminogen activator activity in aortic endothelial cells (27). TFPI is an important regulator of the extrinsic pathway of blood coagulation through its ability to inhibit factor Xa and factor VIIa-tissue factor activity (28, 29). We also investigated the expression of human ryudocan by using an immunohistochemical method utilizing a specific anti-human ryudocan antibody.

EXPERIMENTAL PROCEDURES

Materials—Heparin, HS from bovine kidney, type A chondroitin sulfate (CS) from whale cartilage, chondroitin ABC lyase, fibronectin, type I collagen, bFGF, epidermal growth factor, insulin-like growth
factor, and rabbit anti-human insulin-like growth factor IgG were all purchased from Seikagaku Co. (Tokyo). Human antithrombin III (ATIII) and rabbit anti-human ATIII antibody were provided by Hoechst Japán Ltd. (Tokyo). Rabbit IgGs of anti-human collagen (type I) and anti-human epidermal growth factor were from Cosmo Bio (Tokyo). Chymically synthesized human MK, which has neurite promoting activity, was purchased from Peptide Institute (Suita, Japan). Granulocyte colony-stimulating factor (CSF), granulomonocyte CSF, and interleukin-3 were supplied by Kirin Brewery Co. (Tokyo). Monocoy CSF and rabbit anti-human monocoy CSF IgG were obtained from Morinaga Co. (Tokyo). Rabbit IgGs of anti-erythropoietin, mouse monocoy CSF and rabbit anti-human monocoy CSF were from Nacalai Tesque, Inc. (Kyoto, Japan). Granulocyte colony-stimulating factor, and anti-human epidermal growth factor were from Cosmo Bio (Tokyo).

For ligand binding assay, a solid-phase ligand binding assay was performed as described above. After enzyme treatment, samples were subjected to SDS-PAGE and analyzed by autoradiography as described above.

Radioiodination—Proteins were labeled with [125I]iodide at a specific activity of 50 mCi/ml. Protein concentrations were determined by the Bradford (49) method using the Coomassie Brilliant Blue protein assay kit. The A260 measurements for 50 μl of sample were carried out in a DU7500 spectrophotometer (Beckman Instruments) with BSA as a standard. Purified ryudocan was radiolabeled with Na125I by the chloramine-T method as described previously (1). The radiolabeled complexes were separated from free Na125I by gel filtration through a PD-10 column and analyzed by SDS-PAGE and autoradiography as described above. The radiolabeled species were stored at −70°C until use.

Approximately 200 μg of purified human ryudocan was incubated with 200 μl of 0.1% BSA/PBS containing 0.05% Tween 20. After washing five times with distilled water, the wells were incubated with 0.2 wt% o-phenylenediamine dihydrochloride and 0.01% (v/v) H2O2 in 75 mM phosphate-buffered saline (PBS; 0.137 mM NaCl, 2.7 mM KCl, 0.8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4) containing 0.1% BSA (0.1% BSA/PBS), the wells were incubated with 3% BSA in PBS for 4 h at room temperature to block nonspecific binding sites. The wells were rinsed three times with 0.1% BSA/PBS and incubated with 125I-radiolabeled ryudocan (3000–7000 cpm/well) in 100 μl of 0.1% BSA/PBS at 4°C overnight. After three rinses with 0.1% BSA/PBS to remove unbound ligand, the wells were dried and individually separated. The amount of bound radiolabeled ryudocan in each well was quantified with an Aloka γ-counter (Aloka Co. Ltd., Tokyo).

To assure that the applied proteins stuck to the wells, we performed an immunodetection assay as follows. After coating with the proteins (0.5 μg/well) and blocking nonspecific binding sites as described above, the wells were sequentially incubated with a specific rabbit antibody against each ligand and a peroxidase goat anti-rabbit IgG antibody (Dako Japan Co. Ltd., Kyoto, Japán) in 0.1% BSA/PBS containing 0.05% Tween 20. After washing five times with distilled water, the wells were incubated with 0.2 wt% o-phenylenediamine dihydrochloride and 0.01% (v/v) H2O2 in 75 mM phosphate-buffered saline (PBS; 0.137 mM NaCl, 2.7 mM KCl, 0.8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4) containing 0.1% BSA (0.1% BSA/PBS), the wells were incubated with 3% BSA in PBS for 4 h at room temperature to block nonspecific binding sites. The wells were rinsed three times with 0.1% BSA/PBS and incubated with 125I-radiolabeled ryudocan (3000–7000 cpm/well) in 100 μl of 0.1% BSA/PBS at 4°C overnight. After three rinses with 0.1% BSA/PBS to remove unbound ligand, the wells were dried and individually separated. The amount of bound radiolabeled ryudocan in each well was quantified with an Aloka γ-counter (Aloka Co. Ltd., Tokyo).

To verify that unlabeled ryudocan exhibits similar binding properties, we also tested the competitive ability of unlabeled ryudocan against 125I-labeled protein. We used a solid-phase ligand binding assay as described above. Following the addition of [125I]iodide, we performed the assay in the presence of increasing concentrations of unlabeled protein.

Radioiodination—Proteins were labeled with [125I]iodide at a specific activity of 50 mCi/ml. Protein concentrations were determined by the Bradford (49) method using the Coomassie Brilliant Blue protein assay kit. The A260 measurements for 50 μl of sample were carried out in a DU7500 spectrophotometer (Beckman Instruments) with BSA as a standard. Purified ryudocan was radiolabeled with Na125I by the chloramine-T method as described previously (1). The radiolabeled complexes were separated from free Na125I by gel filtration through a PD-10 column and analyzed by SDS-PAGE and autoradiography as described above. The radiolabeled species were stored at −70°C until use.

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Radioiodination—Proteins were labeled with [125I]iodide at a specific activity of 50 mCi/ml. Protein concentrations were determined by the Bradford (49) method using the Coo
peroxidase-conjugated streptavidin (Histofine SAB-PO kits, Nichirei Co., Tokyo) according to the manufacturer's protocols. After washing with Tris-buffered saline, the tissue sections were incubated in 0.05M sodium acetate/acetic acid buffer, pH 5.0, containing 0.02% (w/v) 3-amino-9-ethylcarbazole and 0.014% (w/v) H$_2$O$_2$ and allowed to react for 5–10 min. Sections were finally counterstained with hematoxylin and mounted in glycerin/gelatin solution (glycerin, 20% gelatin solution of 1:1). As a negative control, PBS was used instead of rabbit anti-human ryudocan antibody. To assure the specificity of the signals, we also used an excess amount of Ryu2 peptide to compete with the native ryudocan molecule in the specific binding of anti-human ryudocan IgG. Thus, we incubated rabbit anti-human ryudocan IgG with an excess amount of Ryu2 peptide (molar ratio of 3000:1) for 20 h at 4 °C prior to treatment of samples following the immunostaining procedures as described above.

RESULTS

Purification and Characterization of Human Ryudocan from EAhy926 Cells—We purified human ryudocan from EAhy926 cells by using a combination of anion-exchange and immuno-affinity chromatography (see “Experimental Procedures”). The overall yields of human ryudocan from conditioned media of EAhy926 cells for the entire purification procedure averaged...
These results were similar to previously reported purification yields of rat ryudocan from rat fat pad microvascular endothelial cells (1).

To determine the purity of the proteoglycan preparation and to identify the attached glycosaminoglycan chains, this material was subjected to enzymatic degradation. After radiolabeling with \( ^{125}\text{I} \), the human ryudocan preparation was treated with purified Flavobacterium heparitinase or chondroitin ABC lyase and analyzed by SDS-PAGE through a 4–15% gradient (Fig. 1). Autoradiography of the resulting material showed a diffuse band with an apparent molecular mass of 160 kDa. Treatment of the sample with purified Flavobacterium heparitinase resulted in a single distinct band with an apparent molecular mass of 30 kDa. This corresponded to the previously described core protein size of rat ryudocan from microvascular endothelial cells (1). In contrast, chondroitin ABC lyase treatment did not affect the migration of the iodinated 160-kDa molecule. These data indicate that the purification procedure yielded pure human ryudocan, which was predominantly composed of heparan sulfate GAG chains.

Ryudocan Binding to Potential Ligands—The binding of \( ^{125}\text{I} \)-labeled human ryudocan to potential ligands was examined by using the solid-phase assay as described under “Experimental Procedures.” Microtiter wells (precoated with a 1 \( \mu \)g/well con-
FIG. 7. Ryudocan-bFGF, -MK, and -TFPI Binding
centration of the proteins) were incubated with $^{125}$I-labeled human ryudocan. After washing the wells, the amount of bound radioactive material was determined. The results indicate that bFGF, MK, and TFPI exhibited significant $^{125}$I-ryudocan binding (Fig. 2). In contrast, fibronectin, type I collagen, epidermal growth factor, insulin-like growth factor, granulocyte CSF, monocyte CSF, granulomonocyte CSF, interleukin-3, and ATIII showed no significant affinity for ryudocan, even if the concentrations of these proteins were increased to 5 μg/well (data not shown).

To assure that the applied proteins stuck to the wells, we performed an immunodetection assay as described under “Experimental Procedures.” The significant signals of absorbance at A$_{405}$ were obtained from all ligands tested, compared with those from the BSA control (data not shown). In addition, we measured the amount of protein remaining in the wells after removal of ligand solution by using a $^{125}$I-labeled tracer for each protein. The amounts of the remaining ligands in the wells ranged from 6.0 to 20.7%. These data suggest that a lack of ryudocan binding could not result from a failure of the applied proteins to stick to the wells.

We also performed a competition assay to verify that unlabeled ryudocan exhibits similar binding properties as described under “Experimental Procedures.” Unlabeled ryudocan inhibited dose-dependently the binding of $^{125}$I-ryudocan to bFGF, MK, and TFPI (Fig. 3). Presumably, due to excess of coated ligands, a relatively large amount of unlabeled ryudocan would be needed to block the binding of $^{125}$I-ryudocan. Thus, the observed $^{125}$I-ryudocan binding might not be a consequence of the iodination reaction.

To investigate the involvement of ryudocan GAG chains in ligand binding, we digested the GAG chains of $^{125}$I-ryudocan with purified Flavobacterium heparitinase or chondroitin ABC lyase prior to the solid-phase binding assay (Fig. 4). Heparitinase treatment destroyed the ability of $^{125}$I-ryudocan binding to bFGF, MK, and TFPI. In contrast, chondroitin ABC lyase treatment did not affect $^{125}$I-ryudocan binding to these potential ligands. These data indicate that the HS chains, not the core protein of ryudocan, are responsible for binding bFGF, MK, and TFPI.

We also tested the inhibition of ryudocan binding by heparin, HS, and CS (Fig. 5). We were found that heparin distinctly inhibited the binding of $^{125}$I-ryudocan to bFGF, MK, and TFPI. HS also moderately inhibited $^{125}$I-ryudocan binding to these molecules, but at high concentrations. In contrast, CS had no inhibitory effect on $^{125}$I-ryudocan binding to these ligands.

We examined the concentration-dependent binding of ryudocan to bFGF, MK, and TFPI by a solid-phase assay as described under “Experimental Procedures.” We found that the binding of ryudocan to bFGF, MK, and TFPI was saturable (Fig. 6). Scatchard analyses showed that the apparent K$_d$ values were 0.50 nM for bFGF, 0.30 nM for MK, and 0.74 nM for TFPI (Fig. 6, inset).

**DISCUSSION**

Ryudocan is one of the syndecan family members that are type I integral membrane HSPGs. HS binds a variety of proteins, including peptide growth factors, extracellular matrix components, cell adhesion molecules, lipolytic enzymes, protease inhibitors, and circulating lipoproteins. Syndecan, the best characterized molecule in its family, has been shown to selectively bind a variety of extracellular matrix molecules, suggesting that syndecan may be an extracellular matrix receptor (5–9).

Recently, it was shown that cell-surface HS appears to be required for the binding of bFGF to its high affinity receptor (15). Syndecan from mammary epithelial cells bound bFGF via its HS chains and was considered a good candidate for a cell-surface HSPG low affinity receptor (11). However, overexpression of syndecan at the surface of NIH 3T3 cells has been shown to increase fibronectin binding and yet inhibit bFGF-induced cell proliferation (37). It was also reported that syndecan as well as glypicin and fibroglycan block heparin-dependent bFGF receptor binding due to competitive inhibition (38). In addition, perlecain, a large basal lamina proteoglycan, has been identified as a major candidate for a bFGF low affinity accessory receptor as well as an angiogenic modulator by virtue of its differential HS structure (39). Nevertheless, it is still possible that bFGF high affinity interactions in vivo require cooperative effects between these cellular HSPGs. In this study, we showed that ryudocan has a specific binding affinity for bFGF via its HS chains, with an apparent K$_d$ of 0.50 nM. We also demonstrated that ryudocan is distinctly expressed in several fibrous tissues as well as in fetal lung capillaries and endocardium. These data suggest that ryudocan may participate in fibroblast growth and fetal angiogenesis through the bFGF interaction either as a low affinity receptor or as an inhibitor of pathogenesis or cellular development.

MK showed the highest affinity (apparent K$_d$ = 0.30 nM) for ryudocan among the three ligands tested. MK promotes both neurite outgrowth and the survival of various embryonic neurons, and the neurite promoting activity is in the COOH-terminal half of the MK molecule, which has heparin binding activity (30). MK has ~50% sequence identity to pleiotrophin (40), also called heparin binding growth-associated molecule (41). MK and pleiotrophin have similar functions and constitute a new family of heparin-binding proteins involved in regulation of cellular growth and differentiation (42). The neurite

**Fig. 7. Immunohistochemical Analysis of Ryudocan Expression**—To investigate the localization of ryudocan expression, we analyzed several tissues by immunohistochemical staining as described under “Experimental Procedures.” We found that cytotrophoblasts of placental villi as well as Schwann cells of peripheral nerve tissues were distinctly stained (Fig. 7, A and B). Interestingly, the fetal pulmonary endothelium and endocardium, but not the adult counterparts, were stained clearly (Fig. 7, C and D). We also found that human ryudocan was significantly expressed in some pathological tissues, including fibrous regions of renal Bowman’s capsules and coronary atheromatous plaques (Fig. 7, E and F).

To demonstrate the specificity of the signals, we used Ryu2 peptide as a competitor with the native ryudocan molecule as described under “Experimental Procedures.” The signals of cytotrophoblasts as well as the fetal pulmonary endothelium faded away when rabbit anti-human ryudocan IgG was incubated with an excess amount of Ryu2 peptide prior to immunostaining (Fig. 7, G and H). These results thus suggest that the specific signals would be specific for the human ryudocan molecule.
promoting activity was strongly inhibited by heparin and only weakly by HS.\(^2\) This mode of inhibition is quite similar to that of ryudocan binding to MK described in this paper. We also demonstrated in this report that purified ryudocan possesses significant affinity for TFPI, but not for ATIII. These data suggest that human ryudocan may have an anticoagulant activity through its TFPI interaction. This may be one physiologic function of ryudocan in the placental villus cytotrophoblasts.

The variation in structure and binding affinity of cell-surface HS on syndecan is a differentiated characteristic of each cell type (46). This enables cells to respond to the specific HS-binding effectors in the cellular microenvironment. The attachment of different numbers of HS and CS chains to syndecan family members may alter interactions with specific proteins and hence modify the biological function of these components. It has been reported that TFPI is detected specifically in macrophages in the villi of term placenta (44) and that heparin enhances the rate of factor Xa inhibition by recombinant TFPI in the presence of Ca\(^{2+}\) (45). In this study, we demonstrated that purified ryudocan from EAHy926 cells possesses significant affinity for TFPI, but not for ATIII. These data suggest that human ryudocan may have an anticoagulant activity through its TFPI interaction. This may be one physiologic function of ryudocan in the placental villus cytotrophoblasts.

Several investigators have shown that HSPG is present on the endothelial cell surface and functions as an anticoagulant (43). Interestingly, ryudocan was originally isolated from rat microvascular endothelial cells as an anticoagulant HSPG (1, 2). Immunohistochemical analysis revealed that ryudocan is distinctly expressed in placental cytotrophoblasts as well as in fetal lung capillaries. Placental tissues are known to be resources for tissue factor involved in blood coagulation, and placental dysfunction may cause disseminated intravascular coagulopathy. It has been reported that TFPI is detected specifically in macrophages in the villi of term placenta (44) and that heparin enhances the rate of factor Xa inhibition by recombinant TFPI in the presence of Ca\(^{2+}\) (45). In this study, we demonstrated that purified ryudocan from EAHy926 cells possesses significant affinity for TFPI, but not for ATIII. These data suggest that human ryudocan may have an anticoagulant activity through its TFPI interaction. This may be one physiologic function of ryudocan in the placental villus cytotrophoblasts.

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\(^2\) N. Kaneda, unpublished data.