Syndecan-4 Deficiency Impairs Focal Adhesion Formation Only under Restricted Conditions*

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Two domains of fibronectin deliver two different but cooperative signals required for focal adhesion formation. The signal from the cell-binding domain is mediated by integrins, whereas the signal from the heparin-binding domain is recognized by heparan sulfate proteoglycans, of which syndecan-4 has been hypothesized to be involved in focal adhesion formation. We generated mice deficient in syndecan-4 to study its role directly. Even in fibroblasts from syndecan-4-deficient mice, focal adhesions were formed, and actin fibers terminated normally at focal adhesions when they were cultured on coverslips coated with fibronectin or with a mixture of its cell-binding and heparin-binding fragments. However, when the cells were cultured on the cell-binding fragment and the heparin-binding fragment was added to the medium, focal adhesion formation was impaired in the syndecan-4 null fibroblasts as compared with that in wild-type cells. Therefore, syndecan-4 is essential for promoting focal adhesion formation only when the signal of the heparin-binding domain of fibronectin is delivered as a soluble form, most probably from the apical surface. When the signal is delivered as a substratum-bound form, other molecule(s) also participate(s) in the signal reception.

Interactions between cells and extracellular matrices are highly important in the regulation of various biological processes such as development, growth, and repair. Focal adhesions are macromolecular complexes found at the sites of cell adhesion to extracellular matrices (1, 2). Focal adhesions are linked to actin stress fibers and also serve as signaling complexes involved in triggering intracellular signaling cascades.

When cells are plated on fibronectin, focal adhesion formation requires two independent signals delivered from the cell-binding domain and the heparin-binding domain of the molecule (2–4). The signal from the RGD-containing cell-binding domain is mediated through integrins (5, 6), whereas the heparin-binding domain is recognized by heparan sulfate proteoglycan(s) (2–4). Among these, syndecan-4 (also called ryudocan or amphiglycan; see Refs. 7–9) is believed to be important, because this transmembrane protein is a component of focal adhesions (10, 11) and antibodies directed against the ectodomain of syndecan-4 cooperate with the cell-binding fragment of fibronectin to form focal adhesions (12). To evaluate the precise role of syndecan-4 in focal adhesion formation, we generated mice deficient in the syndecan-4 gene (Synd4)† and examined fibroblasts from these animals.

EXPERIMENTAL PROCEDURES

Materials—Intact human fibronectin and its cell-binding fragment (CBF) were purchased from Wako Chemical Co. Heparin-binding fragment of fibronectin (HBF), tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin, anti-vinculin monoclonal antibody, fluorescein isothiocyanate-conjugated anti-mouse IgG antibody, and TRITC-conjugated anti-rabbit IgG antibody were obtained from Sigma-Aldrich. Anti-syndecan-4 ectodomain antibody (@-Synd4) was prepared as described (13), and anti-syndecan-4 cytoplasmic domain antibody was a gift of Dr. N. W. Shworak (14). Rabbit anti-mouse fibronectin antibody was purchased from Biogenes.

Generation of Targeted Mice—The syndecan-4 targeting vector was constructed from a basic targeting vector (pTNTneo (polyoma virus thymidine kinase gene promoter and neomycin resistance gene) and DTA (diphtheria toxin fragment A gene) (15) and the Synd4 fragments from the AMRGI clone (16). To delete 2452 bases containing exons II and III and part of exon IV of Synd4 (HindIII–ApoI sites, Fig. 1A), a 2.3-kb BamHI/HindIII fragment was used as the 5' arm, and a 4.4-kb ApaI/Smal fragment was used as the 3' arm (Fig. 1A). Generation of targeted ES cells and blastocyst injection were performed as described (15). The male chimeric mice were mated with C57BL/6J female mice, and F1 mice, with the homologously recombined genome were mated with each other to obtain the null mutant mice. The mutant (Synd4+/−) or wild-type (Synd4+/+) embryos were obtained by crossing 2 Synd4+/− female mice and F1 Synd4+/− mice.

Southern, Northern, and Western Blot Analyses—Southern blot analysis was performed as described previously (15). After digestion with BamHI and hybridization with an external probe (a 692-base pair BamHI/BglII fragment; Fig. 1A), the homologously recombined genomic DNA gave a 4-kb band, whereas the wild-type DNA gave an 11-kb band (Fig. 1, A and B). Northern blot analysis was performed as described previously, using 32P-labeled probes of the syndecan family members (13). The proteoglycan fraction was prepared, and Western blot analyses were performed as described previously (13).

Coating of Coverslips—Glass coverslips (24 × 24 mm, Matsunami) were coated with fibronectin or other proteins dissolved in Dulbecco's phosphate-buffered saline (PBS) at 4 °C overnight. The coverslips were washed with PBS three times, incubated with 1% heat-denatured bovine serum albumin in PBS at 37 °C for 30 min, and then washed again with PBS three times.

Cell Culture—Fibroblasts were obtained by mincing three embryos of Synd4−/− or Synd4+/+ on day 13.5 of gestation. After two or three passages, the cells were stocked in liquid nitrogen and used between the second and fifth passages from the stocks. Unless otherwise specified, the cells were cultured in 10% fetal calf serum (FCS)/Dulbecco's modi-
fied Eagle’s medium (DMEM) at 37 °C under 5% CO₂. Subconfluent fibroblasts were trypsinized and then harvested with 10% FCS/DMEM. The cells were rinsed twice with DMEM and then suspended in DMEM (2 × 10⁴ cells/ml). Four hundred µl of the cell suspension was seeded onto the coverslips.

Focal Adhesion Formation in Response to the Heparin-binding Fragment of Fibronectin or Anti-syndecan-4 Ectodomain Antibody—The experimental procedures were as described by Woods et al. (3, 4) with some modifications. Briefly, subconfluent fibroblasts were treated with 10 µg/ml cycloheximide (Nacalai Tesque) for 2 h. Cycloheximide was present throughout all steps to prevent endogenous fibronectin secretion. The cells were trypsinized, harvested, and seeded onto coverslips according to the procedure described above; this was followed by incubation for 2 h. Then, DMEM was changed to fresh DMEM or to fresh DMEM with added proteins. The cells were further incubated for 2 h and then fixed and examined.

Assay of Focal Adhesion Formation—The fixation and immunocytochemical staining were performed as described by Woods and Couchman (10). Actin fibers were stained with TRITC-conjugated phalloidin. A laser scanning confocal imaging system (Bio-Rad) was used for observation and for recording immunofluorescence. More than 15 fields at a magnification of ×200 were recorded, and then more than 100 cells were scored in each assay. In the present study, we considered that focal adhesions were formed if, even in a part of a cell, the cell contained streaks stained with anti-vinculin antibody where actin fibers terminated. Cells that did not spread sufficiently were excluded for scoring. There were no differences in the numbers of attached and spread cells between Synd4(+/+) and Synd4(−/−) cells; about 80% of seeded cells attached, and more than 90% of them spread in all experiments. Observations, recording, and scoring were performed in a blind manner. Experiments were performed four times using two independent fibroblast stocks. Statistical analyses were performed with StatView 4.5 (Abacus).

RESULTS AND DISCUSSION

Production of Mice Lacking Synd4—The coding region of Synd4 is distributed over 5 exons (16). We deleted exons II and III and part of IV, which correspond to the ectodomain with three putative glycosaminoglycan attachment sites, by replacing them with the neomycin resistance gene, employing the homologously recombined genomic DNA. DNA was isolated from the tails of mice of the indicated genotype. The homologously recombined genomic DNA gave a 4.3 kb band, whereas the wild-type DNA gave an 11-kb band. C, Northern blot analysis. Total RNA (10 µg) was isolated from the kidney. The arrow indicates the size of syndecan-4 mRNA. The lower panel shows the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. D, Western blot analysis of proteoglycans isolated from the kidney; they were separated by SDS-polyacrylamide gel electrophoresis and reacted with anti-syndecan-4 ectodomain antibody. The arrows indicate a broad band of around 200 kDa corresponding to syndecan-4 before heparitinase digestion and a shifted band with a molecular mass slightly higher than 31 kDa after heparitinase digestion. E, Western blot analysis of lysates from fibroblasts to exclude the possible presence of a mutant syndecan-4 protein without the ectodomain in Synd4(−/−) cells. The lysates were concentrated by Centricon YM-3, separated by SDS-polyacrylamide gel electrophoresis, and reacted with anti-syndecan-4 cytoplasmic domain antibody (lanes 1 and 2) or normal rabbit serum (lane 3). The positive control, lysate from Synd4(+/-) fibroblasts (lane 1), was digested with heparitinase. A band of more than 45 kDa was the non-specifically stained one, because it reacted also with normal rabbit serum (lane 3). The arrow indicates the size of the syndecan-4 core protein.

FIG. 1. Strategy for deletion of Synd4 and Southern, Northern, and Western blot analyses. A, exons II and III and part of exon IV were replaced with the neomycin resistance gene (Neo). DTA, diphtheria toxin fragment A gene; II, HindIII; B, BamHI; K, KpnI; Bg II; A, ApaI; Ev, EcoRV; Sm, SmaI; Xh, Xhol; Ex, exon. B, Southern blot analysis after BamHI digestion. An external probe (Probe in A) was used. DNA was isolated from the tails of mice of the indicated genotype. The homologously recombined genomic DNA gave a 4.3 kb band, whereas the wild-type DNA gave an 11-kb band. C, Northern blot analysis. Total RNA (10 µg) was isolated from the kidney. The arrow indicates the size of syndecan-4 mRNA. The lower panel shows the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. D, Western blot analysis of proteoglycans isolated from the kidney; they were separated by SDS-polyacrylamide gel electrophoresis and reacted with anti-syndecan-4 ectodomain antibody. The arrows indicate a broad band of around 200 kDa corresponding to syndecan-4 before heparitinase digestion and a shifted band with a molecular mass slightly higher than 31 kDa after heparitinase digestion. E, Western blot analysis of lysates from fibroblasts to exclude the possible presence of a mutant syndecan-4 protein without the ectodomain in Synd4(−/−) cells. The lysates were concentrated by Centricon YM-3, separated by SDS-polyacrylamide gel electrophoresis, and reacted with anti-syndecan-4 cytoplasmic domain antibody (lanes 1 and 2) or normal rabbit serum (lane 3). The positive control, lysate from Synd4(+/-) fibroblasts (lane 1), was digested with heparitinase. A band of more than 45 kDa was the non-specifically stained one, because it reacted also with normal rabbit serum (lane 3). The arrow indicates the size of the syndecan-4 core protein.

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Role of Syndecan-4 in Focal Adhesion Formation

Blasts were cultured in DMEM for 3 hours on coverslips coated with 10 μg/ml fibronectin. F. Immunocytochemical localization of endogenous fibronectin (red) and its muconcytochemical staining of vinculin and actin fibers in Synd4(-/-) fibroblasts. E. Double staining of vinculin and actin fibers in Synd4(+/+) fibroblasts. F. Immunocytochemical localization of endogenous fibronectin (red) and its relationship to focal adhesion as revealed by anti-vinculin staining (yellow or green streaks) in Synd4(-/-) fibroblasts. Except for F, fibroblasts were cultured in DMEM for 3 h on coverslips coated with 10 μg/ml fibronectin. In F, fibroblasts were cultured in 10% FCS/DMEM for 3 h on coverslips coated with 1 mg/ml gelatin. The FCS used in F was passed through gelatin-Sepharose twice to remove fibronectin.

than 90% of the Synd4(-/-) fibroblasts spread and about 80% of them formed focal adhesions at each point; there were no significant differences between the Synd4(+/+) and Synd4(-/-) fibroblasts (data not shown). Thus, syndecan-4 was not essential for focal adhesion formation and actin fiber formation at focal adhesions.

It has been reported that syndecan-4 is colocalized with endogenous fibronectin in focal adhesions (11), although it is not known whether syndecan-4 directs the deposition of fibronectin. Thus, we examined the distribution of endogenous fibronectin and found that syndecan-4 did not influence the distribution of endogenous fibronectin, present at or near focal adhesions in both Synd4(-/-) (Fig. 2F) and Synd4(+/+) fibroblasts (data not shown).

Roles of Syndecan-4 in Signaling from Heparin-binding Fragment of Fibronectin—To evaluate the effects of the two fragments of fibronectin, fibroblasts were treated with cycloheximide to suppress the synthesis of endogenous fibronectin. Even under these conditions, Synd4(-/-) fibroblasts formed focal adhesions on fibronectin-coated coverslips with efficiency similar to that of Synd4(+/+) fibroblasts (Fig. 3, columns 1 and 2). Both Synd4(-/-) and Synd4(+/+) fibroblasts poorly formed focal adhesions on coverslips coated only with the CBF (Fig. 3, columns 3 and 4). However, when the fibroblasts were seeded onto coverslips coated with CBF and incubated for 2 h, and then the HBF was added to the medium, the incidence of focal adhesion formation increased in Synd4(+/+) but not in Synd4(-/-) fibroblasts (Fig. 3, columns 5 and 6). When fibroblasts were cultured on coverslips coated with a mixture of CBF and HBF, the efficiency of focal adhesion formation increased in both Synd4(+/+) and Synd4(-/-) fibroblasts (Fig. 3, columns 7 and 8). Inhibition of the HBF action by heparin (Fig. 3, columns 9 and 10) confirmed that HBF itself, but not a possible contaminant in the preparation, exerted the effect.

Thus, we concluded that syndecan-4 is essential for receiving the signal from soluble HBF, most probably at the apical surface, but is not essential for signal reception from the substratum-bound HBF.

We next examined the effects of anti-syndecan-4 antibody (a-Synd4). When added instead of HBF, a-Synd4 in either the soluble or substratum-bound form cooperated with CBF to induce focal adhesion formation in Synd4(+/+) fibroblasts (Fig. 3, columns 11 and 13; Ref. 12). However, in Synd4(-/-) fibroblasts, a-Synd4 added not only in the medium (Fig. 3, column 12) but also as a substratum-bound form (Fig. 3, column 14) did not exert this effect. a-Synd4 recognizes only the syndecan-4 molecule; however, HBF reacts not only with syndecan-4 but also with other molecules. Thus, the different results obtained by a-Synd4 and HBF lead to two conclusions: 1) Syndecan-4 is confirmed to be involved in the signal reception for focal adhesion formation; 2) some other molecule(s), which may have heparan sulfates, compensate(s) for the loss of syndecan-4 in the signal reception from the substratum-bound HBF.

The molecules that compensate for the loss of syndecan-4 may be other members of the syndecan family. A previous study demonstrated that antibody-induced clustering of syndecan-1 led to the reorganization of actin fibers, although the study did not examine focal adhesion formation (17). Indeed, syndecan-1 mRNA as well as syndecan-2 and -3 mRNAs were strongly expressed in both Synd4(-/-) and Synd4(+/+) fibroblasts; the mRNA levels of the molecules were not different between Synd4(-/-) and Synd4(+/+) fibroblasts (data not shown).

Synd4(-/-) mice showed no macroscopic abnormalities and reproduced normally. Thus, the syndecan-4 requirement for a soluble form of HBF to promote focal adhesion formation is not essential for ontogenesis and reproduction. However, when inflammation or wounding occurs, fibronectin is degraded by proteases (15, 19). It has been also reported that syndecan-4 is expressed in infiltrating macrophages in ischemic areas of myocardial infarction (2), granulation tissue adjacent to skin wounds (21), and vascular smooth muscle cells at injured arteries (22). Furthermore, it has been suggested that focal adhesions may be involved in cell migration (2). Thus, syndecan-4 might participate in the processes of inflammation and wound recovery through focal adhesion formation and actin fiber organization in response to the signal from the heparin-binding domain of fibronectin.
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