

The Cysteine-rich Domain of Human ADAM 12 Supports Cell Adhesion Through Syndecans and Triggers Signaling Events that Lead to β 1 Integrin-dependent Cell Spreading

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Abstract. The ADAMs (a disintegrin and metalloprotease) family of proteins is involved in a variety of cellular interactions, including cell adhesion and ectodomain shedding. Here we show that ADAM 12 binds to cell surface syndecans. Three forms of recombinant ADAM 12 were used in these experiments: the cysteine-rich domain made in *Escherichia coli* (rADAM 12-cys), the disintegrin-like and cysteine-rich domain made in insect cells (rADAM 12-DC), and full-length human ADAM 12-S tagged with green fluorescent protein made in mammalian cells (rADAM 12-GFP). Mesenchymal cells specifically and in a dose-dependent manner attach to ADAM 12 via members of the syndecan family. After binding to syndecans, mesenchymal cells spread and form focal adhesions and actin stress fibers. Integrin β 1 was responsible for cell spreading be-

cause function-blocking monoclonal antibodies completely inhibited cell spreading, and chondroblasts lacking β 1 integrin attached but did not spread. These data suggest that mesenchymal cells use syndecans as the initial receptor for the ADAM 12 cysteine-rich domain-mediated cell adhesion, and then the β 1 integrin to induce cell spreading. Interestingly, carcinoma cells attached but did not spread on ADAM 12. However, spreading could be efficiently induced by the addition of either 1 mM Mn^{2+} or the β 1 integrin-activating monoclonal antibody 12G10, suggesting that in these carcinoma cells, the ADAM 12-syndecan complex fails to modulate the function of β 1 integrin.

Key words: ADAM • syndecan • integrin • cell adhesion • receptor cross-talk

Introduction

Control of cell adhesion is important in several biological phenomena, including embryonal development and tumor cell invasion and metastasis (Damsky and Werb, 1992; Yamada and Geiger, 1997; Couchman and Woods, 1999; Giancotti and Ruoslahti, 1999). Understanding the molecular mechanisms of these processes requires information about a variety of extracellular ligands, their interaction with cell membrane receptors, and the subsequent downstream signal cascade. One class of cell adhesion receptors is the integrins that often work in cooperation with other cell surface molecules. Integrin α 5 β 1-mediated cell adhesion of

cultured fibroblastic cells to fibronectin requires syndecan-4 as a coreceptor (Saoncella et al., 1999), and the cell surface chondroitin sulfate proteoglycan NG2 cooperates with α 4 β 1 in mediating melanoma cell adhesion and spreading (Eisenmann et al., 1999). Similarly, multimolecular receptor adhesion complexes have been described for tetraspan molecules and integrins (Hemler, 1998).

The ADAMs¹ (a disintegrin and metalloprotease) con-

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¹Abbreviations used in this paper: ADAM, a disintegrin and metalloprotease; GFP, green fluorescent protein; rADAM 12-cys, recombinant cysteine-rich domain of human ADAM 12; rADAM 12-DC, recombinant disintegrin-like and cysteine-rich domain of mouse ADAM 12; rADAM 12-GFP, recombinant full-length ADAM 12-S tagged with GFP; RT, reverse transcription; SVMP, snake venom metalloprotease; TDM, triple deletion mutant.

stitute a recently discovered family of cell adhesion receptors (Wolfsberg and White, 1996; Blobel, 1997; Black and White, 1998; Schlöndorff and Blobel, 1999; Stone et al., 1999; Primakoff and Myles, 2000). Over 20 members have been described so far (see <http://www.med.virginia.edu/~jag6n/whitelab.html>). Most members are composed of pro-, metalloprotease, disintegrin-like, cysteine-rich, EGF-like repeat, transmembrane, and cytoplasmic tail domains. One important role of ADAMs relates to their metalloprotease activity (Black and White, 1998; Schlöndorff and Blobel, 1999). A well-studied example is ADAM 17 (TACE), which functions in cell surface shedding of a number of growth factors, including tumor necrosis factor- α (TNF α), transforming growth factor- α (TGF α), and Notch (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998; Brou et al., 2000). Another important role of the ADAMs relates to cellular interactions, as shown for fertilin α and β (ADAM 1 and 2) in sperm-egg binding and fusion during fertilization (Almeida et al., 1995; Chen et al., 1999a,b). However, the molecular mechanisms of the ADAMs in cellular interactions are not well-understood.

We have recently described the cloning of secreted and transmembrane forms of human ADAM 12 (Gilpin et al., 1998) that are implicated in myoblast fusion (Yagami-Hiromasa et al., 1995) and myogenesis (Gilpin et al., 1998). Human ADAM 12 is an active metalloprotease containing the highly conserved zinc-binding catalytic motif HEXGH-XXGXXHD (Loechel et al., 1998, 1999). ADAM 12 expression is upregulated in cancer, and in several tumors ADAM 12 immunostaining is enriched along the tumor cell surfaces (Iba et al., 1999). Based on this intriguing tissue distribution pattern, we asked whether ADAM 12 was involved in cell adhesion. We found that the recombinant cysteine-rich domain of human ADAM 12 made in *E. coli* (rADAM 12-cys) supports carcinoma cell adhesion but fails to promote cell spreading (Iba et al., 1999). In this study, we explored the molecular mechanisms underlying this type of cell adhesion. We found that mesenchymal cells attach, spread, and form focal adhesions and organize stress fibers in response to ADAM 12, and that both syndecans and integrins are necessary to mediate these processes. Carcinoma cells, on the other hand, bind to syndecans but do not spread or engage β 1 integrins in the ADAM 12-mediated adhesion process. However, they can be induced to spread by addition of either 1 mM Mn²⁺ or the β 1 integrin-activating mAb 12G10.

Materials and Methods

Antibodies

Integrin β 1 function-blocking mAbs rat IgG₁ AIB2 and mouse IgG2b CSAT were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences. Another integrin β 1 function-blocking mAb (clone P5D2) was obtained from Chemicon. The β 1 function-activating mAb 12G10 (Mould et al., 1995) was kindly provided by M. Humphries (University of Manchester, Manchester, UK) and an integrin β 1 mAb (K20) with no reported effect on cell function was obtained from Immunotech. Rat IgG₁ mAb to mouse tetranectin (Wewer et al., 1998) was used as an isotype Ig control for the AIB2. Function-blocking mAbs to integrin α 1 (clone H22B6), α 2 (clone Gi9), and α 5 (clone SAM1) were obtained from Immunotech, and mAbs to integrin α 3 (P1B5) was obtained from Life Technologies. Function-blocking mAb to integrin α 6 (clone 135.13C) was kindly provided by A. Mercurio (Beth Israel Hospital, Harvard, Boston, MA). For immu-

nostaining of focal adhesions, mAb to vinculin (kindly provided by M. Glukhova, Institut Curie, Paris, France) was used. Tetramethyl rhodamine isothiocyanate-phalloidin was obtained from Molecular Probes and used to stain actin. mAbs to syndecan-1 (281-2) (Jalkanen et al., 1985; Liebersbach and Sanderson, 1994) and to glypican-1 (kindly provided by A.D. Lander, University of California Irvine, Irvine, CA) (Litwack et al., 1998) were used to stain transfected ARH-77 cells. For Western blotting, a pan-syndecan antiserum that reacts with all syndecans (kindly provided by A.C. Rapraeger, University of Wisconsin, Madison, WI) (Reinland et al., 1996; Ott and Rapraeger, 1998) and an antiserum to human syndecan-4 (Kojima et al., 1996) were used. To confirm the identity of the recombinant ADAM 12 fragments by Western blotting, two rat mAbs, 14E3 to the cysteine-rich domain (Gilpin et al., 1998) and a newly developed 2F7 to the disintegrin-like domain, were used. The 2F7 mAb was generated and characterized as described previously using recombinant ADAM 12 aa 412-557 polypeptide expressed in *E. coli* as an antigen (Gilpin et al., 1998). IgGs were purified using protein G-Sepharose as described by the manufacturer (Amersham Pharmacia Biotech). Fluorescein- and rhodamine-conjugated antibodies against rabbit, rat, and mouse Igs were purchased from DAKO.

Cell Lines

Except when specified, cell lines were obtained from American Type Culture Collection. The following human cell lines were used: RKO colon carcinoma (kindly provided by A. Mercurio, Boston, MA), MDA-MB-231 breast carcinoma (HTB26), RD rhabdomyosarcoma (CCL 136), SV-HFO human fetal osteoblasts transformed with simian virus 40 virus (Chiba et al., 1993), MG-63 osteosarcoma (CRL 1427), Saos-2 osteogenic sarcoma (HTB 85), HOS osteogenic sarcoma (CRL 1543), MRC-5 lung fibroblast (CCL 171), IMR-90 lung fibroblast (CCL 186), WI-38 lung fibroblast (CCL 75), and CCD 1072SK foreskin fibroblast (CRL 2088). In addition, the murine osteoblastic MC3T3-E1 cell line was used (Sudo et al., 1983). These cell lines were grown in DME, supplemented with glutamax I and 4,500 mg/ml glucose and 10% FBS. The human ARH-77 B-lymphoid cell line and a series of ARH-77-transfected cell lines (Neo, Syn-1, -2, -4, Syn/279t, Syn/Glyp, Glyp/Syn, Glyp-1, and triple deletion mutant [TDM]) (Langford et al., 1998; Liu et al., 1998) were grown in suspension in RPMI 1640 medium with glutamax I supplemented with 5% FBS. All media used in this study were supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, and the cells were grown in Nunc tissue culture flasks (GIBCO BRL) at 37°C in 5% CO₂ in a humidified atmosphere.

Isolation of Primary Osteoblast and Muscle Cells from Mice and Chicken

NMRI mice were obtained from M & B and white Italian \times Isabraum, F1 chicken embryos from Statens Serum Institut.

Primary mouse osteoblast-like cells were isolated from calvaria of newborn (0-3-d-old) NMRI mice using an enzyme mixture containing 0.32 mg/ml collagenase (Sigma C 5894) and 0.25% trypsin (GIBCO BRL) essentially as described (Bellows et al., 1986; Otto et al., 1996). The cells were grown in DME and 10% FBS. The osteogenic capacity of the cells was tested by their ability to deposit minerals. In brief, the cells were grown in above standard medium supplemented with 50 μ g/ml ascorbic acid (Sigma A 2147) and 10 mM β -glycero-phosphate (Sigma G 9891). Mineralization was observed after 10-14 d, and mineralized foci were stained by Alizarin red S (Sigma A 5533) after 21 d in culture.

Primary mouse muscle cells were isolated from the hindlimb muscle of newborn (0-3-d-old) mice using an enzyme mixture of 0.15% trypsin and 0.1% dispase (both enzymes from GIBCO BRL) essentially as described (Allen et al., 1984; DiMario and Strohman, 1988; Partridge, 1997). The cells were cultured in DME with 20% FBS and 2% chick embryo extract. Subconfluent cell cultures were passaged and some cultures were changed to DME with 2% horse serum to confirm the ability of the myoblasts to differentiate and form contracting myotubes.

Primary chicken muscle cell cultures were prepared from 11-d-old chick embryo breast muscle using 0.25% trypsin essentially as described (Bischoff and Holtzer, 1969; Knudsen and Horwitz, 1977).

Isolation of Normal and β 1-Deficient Chondroblasts from Mice Carrying a Conditional Null Mutation in the β 1 Integrin Gene

Chondroblast cell lines with a deletion of the β 1 integrin gene were estab-

lished using mice with a loxP-tagged (floxed) $\beta 1$ integrin gene (Potocnik). Chondroblasts were isolated from 3-d-old pups of homozygously floxed mice and immortalized by infection with a retrovirus transducing a heat-labile variant of the simian virus 40 large T antigen (kindly provided by P. Sharp, Massachusetts Institute of Technology, Cambridge, MA). These $+^{c/+^{c}}$ cells were subsequently infected with an adenovirus transducing the cre-recombinase (Anton and Graham, 1995), resulting in excision of the $\beta 1$ integrin gene in infected cells. $\beta 1$ integrin-null cells ($-/-$) were purified by FACS and further cultured. FACS sorted $\beta 1$ integrin-expressing cells, in which no or only one allele of the $\beta 1$ integrin gene was deleted, served as control cells.

For FACS analysis, cells were briefly trypsinized, washed with growth medium, and resuspended in ice-cold PBS containing 1% BSA. Staining for $\beta 1$ integrin was carried out in the same buffer using FITC-labeled mAb Ha2/5 (PharMingen). Cell sorting was done using a FACSVantage, and analytical stainings were analyzed with a FACSort (Beckton Dickinson).

Morphological and Immunocytochemical Analysis

To detect focal adhesions, cells were fixed at room temperature for 10 min in 4% paraformaldehyde in PBS containing 2 mM $MgCl_2$ and permeabilized with 0.1% Triton X-100 in DME with 15% FBS for 5 min at room temperature. After rinsing and blocking for 30 min in DME with 15% FBS, vinculin antibodies supplied as culture medium supernatant were diluted (1:10) in DME with 2% BSA and incubated with the cells for 30 min followed by a thorough rinse and incubation with secondary antibodies. The actin cytoskeleton was visualized by tetramethyl rhodamine isothiocyanate-phalloidin staining as recommended by the manufacturer. For immunostaining of cell surface syndecan-1 and glypican-1, ARH-77 cell suspensions were rinsed in culture medium and incubated with FITC-conjugated mAb 281 against syndecan-1 or anti-glypican 1 antiserum (10 $\mu g/ml$) followed by a second antibody. Cells were examined using an inverted microscope (Zeiss Axiovert) equipped with phase contrast optics and connected to a PentaMAX chilled charge-coupled device camera (Princeton Instruments, Inc.).

Cell Attachment Assays

The attachment assay was performed as described previously (Iba et al., 1999). Substrates included purified rADAM 12-cys, disintegrin-like domain (rADAM 12-disintegrin), metalloprotease domain (rADAM 12-metallo) made in *E. coli* (Iba et al., 1999), purified recombinant mouse disintegrin-like and cysteine-rich domain (rADAM 12-DC) made in insect cells (Zolkiewska, 1999), and for comparison, mouse laminin-1 from the EHS tumor, human laminin-10/11, and human fibronectin (GIBCO BRL). BSA was used as a negative control. Nunc-Immuno™ 96-well plates with MaxiSorp™ surface (Nunc) were coated with 1–50 $\mu g/ml$ of the substrates in 0.1 M $NaHCO_3$ buffer, pH 9.5, overnight at 4°C, rinsed with PBS, and incubated with 10 mg/ml BSA in PBS for 1 h at 37°C. After a rinse with PBS, 100 μl of the cell suspension ($0.6 \times 10^6/ml$) was added to the wells for 1 h at 37°C in 5% CO_2 in a humidified atmosphere. The wells were rinsed twice in serum-free DME, fixed for 20 min in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, rinsed in PBS and stained with 0.1% crystal violet in 10% methanol (vol/vol). Absorbance was measured with a Multiscan ELISA reader (Labsystems) at 590 nm. A blank value corresponding to an empty well was automatically subtracted. Each assay point was derived from three to six separate wells and repeated three times.

The following experimental conditions were tested: (a) The cells were grown in sulfate-free Fischer's medium containing 10% dialyzed FBS in the presence of 20 mM sodium chlorate (Sigma C 3171), or in both sodium chlorate and 10 mM sodium sulfate as a control (Baeuerle and Huttner, 1986; Keller et al., 1989), for 24 h before the assay. (b) Plates were preincubated with heparin (Sigma H 2149), heparan sulfate (Sigma D 9808), heparin-albumin (Sigma H 0403), chondroitin sulfate A, B, and C, respectively (Sigma C 9819, C 0320, C 4384), or hyaluronic acid (Sigma H 1751) at the concentrations indicated. Alternatively, these compounds were added in solution during the incubation period. (c) The cells were pre-treated for 30 min with 2 mU/ml heparitinase or 50 mU/ml protease-free chondroitinase ABC (#100703 and 100332, respectively; Seikagaku Corp.). (d) The cells were preincubated for 15 min at room temperature with 10 $\mu g/ml$ of function-blocking mAbs to human integrin $\beta 1$ (A2BII or P5D2), integrin $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ (H22B6, Gi9, PIB5, SAM 1, and 135.13C), or as an isotype control, with 10 $\mu g/ml$ IgG₁ rat mAbs to tetranectin (1B11). For experiments with chicken muscle cells, the CSAT mAb was used. (e) Cy-

cloheximide (Sigma C 7698) was added at 25 $\mu g/ml$ for 3 h before the assay period to inhibit protein synthesis. (f) Cytochalasin B (Sigma C 6762) or D (Sigma C 8273) dissolved in DMSO was added at 20 and 0.1 μM , respectively. Unless otherwise indicated, the various reagents were present throughout the 1-h attachment assay period.

To test for induction of cell spreading, a modification of the above mentioned attachment assay was employed. The cells were allowed to attach for 30 min and then Mn^{2+} (0.1 or 1 mM $MnCl_2$) or 10 $\mu g/ml$ of the $\beta 1$ integrin mAbs (12G10 or K20) was added for another 30 min.

Cell Binding Assay with Green Fluorescent Protein-tagged Human ADAM 12-S

An expression construct for full-length human ADAM 12-S fused at the COOH terminus to green fluorescent protein (GFP) was constructed using the pEGFP-N1 from CLONTECH Laboratories, Inc. A DNA fragment containing the entire coding region of ADAM 12-S (pro-, metalloprotease, disintegrin-like, cysteine-rich, EGF-like repeat domains, and the 33-amino acid COOH tail; sequence data available from EMBL/GenBank/DBJ under accession no. AF023477) was generated by PCR using *Pfu* DNA polymerase (Stratagene) and inserted at the EcoRI/SacII sites of pEGFP-N1, such that the COOH terminus of ADAM 12 was in-frame with GFP. A control plasmid for secretion of GFP alone was made by transferring the EcoRI/NotI GFP cassette from pEGFP-N1 to the plasmid pSecTagA (Invitrogen Corp.), resulting in expression of a GFP polypeptide fused at the NH_2 terminus to an Ig κ leader sequence. COS-7 cells were transiently transfected as described previously (Loechel et al., 1998, 1999), the culture medium collected 2 d later, and any cellular debris removed by centrifugation. ARH-77 cells (5×10^5) expressing syndecan-1 or -4 or control ARH-77 cells transfected with an empty vector (neo) were incubated with 1 ml of human ADAM 12-GFP or GFP-containing culture medium. After 1 h incubation at 37°C or at 4°C, the cells were examined by fluorescence microscopy. In other control experiments, heparin was added to the incubation medium or the cells were pretreated with heparitinase.

RNA Extraction and Reverse Transcription (RT)-PCR

Total RNA was purified using the TRIzol reagent (GIBCO BRL). For the reverse transcriptase reaction, the cloned mouse Moloney leukemia virus reverse transcriptase was used as recommended by the manufacturer (Stratagene). Aliquots of cDNA were amplified using the following primers: human syndecan-1 (forward) 5'-GCTCTGGGGATGACTCTGAC-3' and (backward) 5'-GTATTCTCCCCAGGTTTC-3'; human syndecan-2 (forward) 5'-ACATCTCCCTTTGCTAACGGC-3' and (backward) 5'-TAACTCCATCTCCTTCCCCAGG-3'; and human syndecan-4 (forward) 5'-GTCTGGCTCTGGAGATCTGG-3' and (backward) 5'-TGGGGGCTTTCTGTAGATG-3'.

After an initial denaturation at 95°C for 40 s, 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 60 s were carried out. The reaction products were separated on agarose gels. The amplified DNA fragments (syndecan-1, 552 bp; syndecan-2, 539 bp; and syndecan-4, 397 bp) were cloned and sequenced to confirm the identity of the PCR products.

Western Blotting of Syndecans

Extraction, isolation, and Western blotting of syndecans were performed as described previously (Lebakken and Rapraeger, 1996). In brief, RD cells were grown to subconfluence, extracted in 8 M urea, 0.1% Triton X-100, 10 mM Tris-HCl, and 1 mM Na_2SO_4 and incubated with DEAE Sephacel in buffer overnight. The beads were washed and proteins eluted with 1 M NaCl in heparitinase buffer followed by digestion with heparitinase and chondroitinase. The samples were separated by SDS-PAGE and immunoblotting performed using the pan-syndecan antibody.

Biotinylation of Cell Surface Proteins and Isolation of rADAM 12-cys Binding Proteins

To identify which cell surface proteoglycans bind to rADAM 12-cys, an affinity chromatography protocol devised by Hoffman et al. (1998) was employed. In the Hoffman et al. protocol, urea was used in both extraction and running buffer, as the attempt to use either octyl glucoside or Triton X-100 were unsuccessful, apparently because syndecans are insoluble in these lysis buffers and precipitate with the cytoskeleton. RD cells were

washed in PBS, incubated with Ez-Link sulfo NHS-LC-biotin (#21335; Pierce Chemical Co.) at a final concentration of 100 $\mu\text{g/ml}$ for 1 h at room temperature, washed twice with 50 mM ammonium chloride to quench the free biotin, and washed once in PBS. Cell pellets were resuspended and lysed with a dounce homogenizer in 10 mM KCl, 20 mM Tris-HCl, pH 7.4, 0.1% β -mercaptoethanol, 1 mM EDTA (1 ml/ 10^8 cells). After removal of the nuclei by centrifugation at 1,500 g for 5 min, NaCl was added to the supernatant at a final concentration of 0.15 M and the supernatant centrifuged at 50,000 g for 30 min at 4°C. The pellet was solubilized in 1 ml of 8 M urea, 2% Triton X-100, 2 mM PMSF in TBS, pH 7.4, and centrifuged at 14,000 g for 20 min at 4°C to remove insoluble matter. The resulting cell membrane protein solution was incubated with a rADAM 12-cys affinity matrix (rADAM 12-cys coupled to CnBr-activated Sepharose following the manufacturer's instructions) in 6 M urea, 1% Triton X-100, 2 mM PMSF in TBS, pH 7.4, (running buffer) overnight at 4°C under gentle rotation. After a thorough rinse in running buffer, elution was carried out in 20 mM EDTA. The eluted fractions were precipitated with acetone, washed with ethanol, and resuspended, and part of the material was digested with heparitinase (0.1 U/ml) and/or chondroitinase ABC (1 U/ml) at 37°C for 4 h in the presence of aprotinin (2.2 $\mu\text{g/ml}$), leupeptin (1 $\mu\text{g/ml}$), pepstatin A (2 $\mu\text{g/ml}$), and EDTA (5 mM). Undigested and digested material was separated on a 4–12% SDS-PAGE and transferred to Hybond-P (polyvinylidene difluoride) paper (Amersham Pharmacia Biotech). The paper was subsequently blocked in 50 M Tris, 0.5 M NaCl, 0.1% Tween 20, 5% nonfat milk powder, and incubated with peroxidase-conjugated streptavidin at a final concentration of 1 $\mu\text{g/ml}$ or with a specific polyclonal antiserum to human syndecan-4 (Kojima et al., 1996), followed by a peroxidase-conjugated goat anti-rabbit IgG secondary antibody. The blots were developed using the chemiluminescence SuperSignal kit from Pierce Chemical Co.

Purification of Human Syndecan-4 and Solid-phase Binding Assay

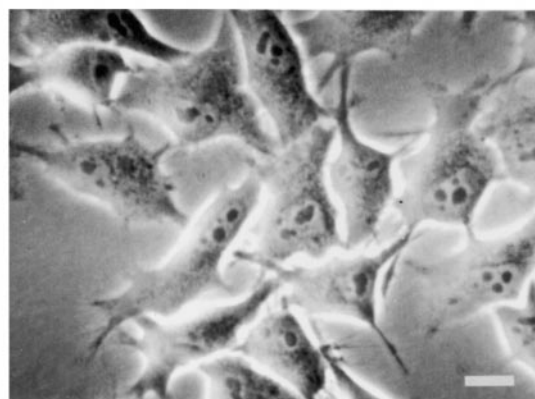
Native human syndecan-4 ectodomain was purified from the culture medium supernatant of endothelium-like Eahy926 cells under non-denaturing conditions and iodinated as described (Kojima et al., 1996). The solid-phase binding assay was used as described previously (Kojima et al., 1996). In brief, microtiter wells were coated with 5 $\mu\text{g/ml}$ of the basic fibroblast growth factor (bFGF) or 50 $\mu\text{g/ml}$ rADAM 12-cys in 100 μl of 50 mM NaHCO₃, pH 9.2, overnight at 4°C, incubated with 1% BSA in PBS at room temperature to block nonspecific binding, and incubated with ¹²⁵I-labeled human syndecan-4 (800 cpm) in 100 μl of 0.1% BSA overnight at 4°C. After rinsing the wells, the amount of bound material was determined using an Aloka γ -counter (Aloka Co. Ltd.).

Results

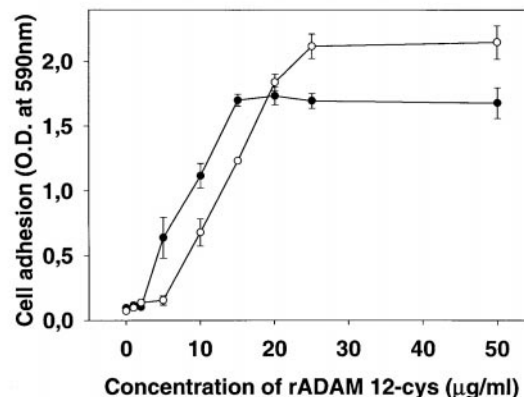
Mesenchymal Cells Attach, Spread, Form Focal Adhesions, and Reorganize Actin Stress Fibers in Response to rADAM 12-cys

In a previous report, we showed that carcinoma cells attach but do not spread on rADAM 12-cys (Iba et al., 1999). Here we examine the molecular mechanism resulting in cell adhesion of a number of different cell types on human rADAM 12-cys made in *E. coli* and mouse rADAM 12-DC made in insect cells. In our assays, we tested cells of osteoblastic origin such as SV-HFO, HOS, Saos-2, MG-63, MC3T3-E1 cell lines, and primary murine osteoblasts; fibroblastic cells such as MRC-5, WI-38, IMR-90, and CCD1072SK; myoblastic cells such as RD cells and primary murine and chicken muscle cells. Each of these mesenchymal cell lines attached and >90% of the cells displayed a spread and flattened morphology on rADAM 12-cys (Fig. 1 A). A similar adhesion pattern was observed when the cells were plated on laminin or fibronectin (not shown). Cell attachment was specific and dose-dependent, and reached a maximum at ~15–25 $\mu\text{g/ml}$ (Fig. 1, B and C). Cells attached efficiently to rADAM 12-DC (Fig. 1 C),

A



B



C

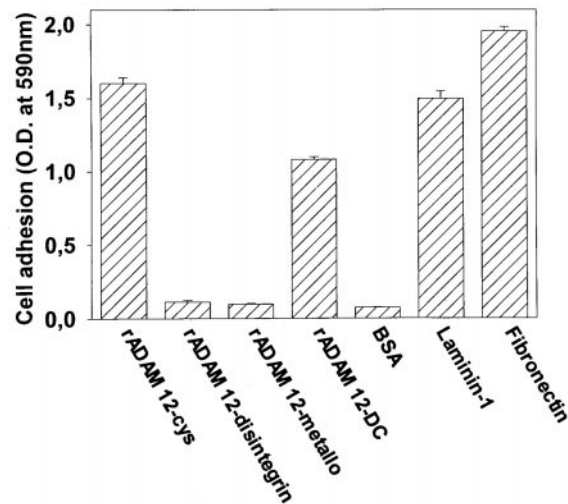


Figure 1. Mesenchymal cell attachment and spreading in response to rADAM 12-cys. (A) Cell attachment of MG-63 cells plated on rADAM 12-cys (20 $\mu\text{g}/\mu\text{l}$). Bar, 16 μm . (B) Dose-dependent cell attachment of SV-HFO (filled circles) and RD cells (open circles) plated on rADAM 12-cys. (C) Cell attachment of the SV-HFO cell line plated on rADAM 12-cys, rADAM 12-disintegrin, rADAM 12-metallo, rADAM 12-DC, laminin-1, and fibronectin (20 $\mu\text{g}/\mu\text{l}$). BSA represents the background value when no substrate was added. Ordinates represent OD values at 590 nm, and abscissa the concentration of the substrate in $\mu\text{g}/\mu\text{l}$ as indicated. Each point represents the mean \pm SEM of triplicate wells.

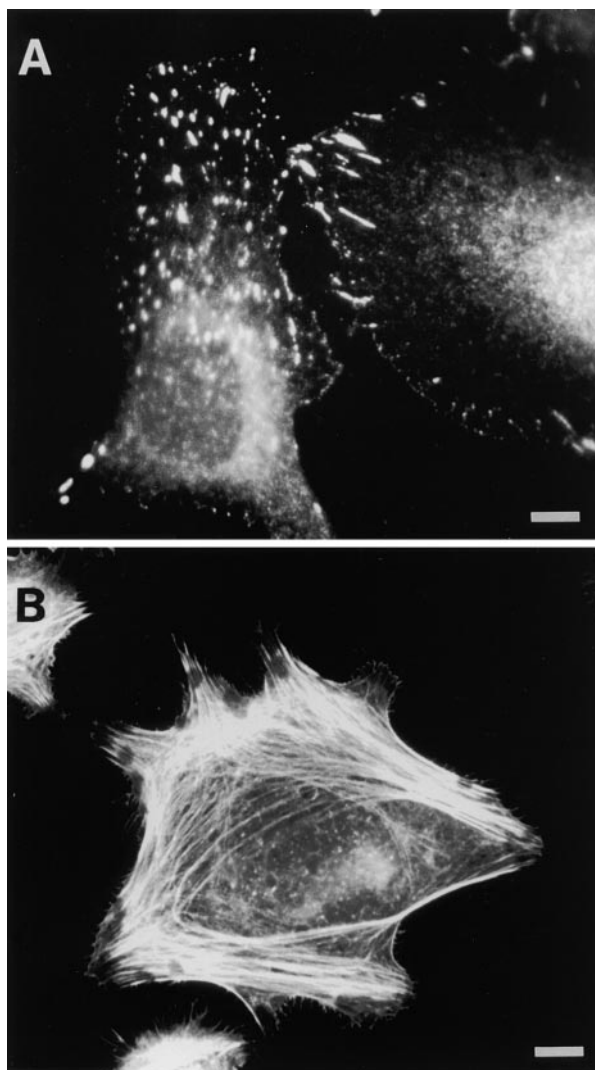


Figure 2. Focal adhesion formation and reorganization of the actin cytoskeleton in response to rADAM-cys. (A) SV-HFO cells plated on rADAM 12-cys and stained with mAbs to vinculin. Numerous distinct focal adhesions are seen. (B) SV-HFO cells plated on rADAM 12-cys and stained with phalloidin to detect F-actin. An elaborate organization of stress fibers is evident. Bars, 6 μm .

but cell spreading appeared less efficient than on rADAM 12-cys at equimolar concentrations. No cell attachment was observed on either the disintegrin-like or the metalloprotease domains made in *E. coli*. (Fig. 1 C). The fully spread mesenchymal cells formed focal adhesions and contained an elaborate actin cytoskeleton when plated on rADAM 12-cys (Fig. 2). Cell spreading and stress fiber formation was decreased by treatment of the cells with cytochalasin B and D, inhibitors of actin polymerization (not shown). These results demonstrate that in contrast to carcinoma cells, which attach but fail to spread, mesenchymal cells attach, spread, form specialized adhesion structures, and reorganize the actin cytoskeleton in response to rADAM 12-cys.

Syndecans Serve as Primary Attachment Receptors for rADAM 12-cys

We next determined which class of cell adhesion receptors mediates adhesion to rADAM 12-cys and rADAM 12-DC. Since we found previously that attachment of carcinoma cells was dependent on heparan sulfate chains (Iba et al., 1999) and that CHO cells deficient in heparan sulfate synthesis (CHO pgsD-677) were unable to attach to rADAM 12-cys (Iba, K., and U.M. Wewer, unpublished results), several compounds interfering with the function of cell surface heparan sulfates were tested in adhesion assays (Fig. 3 A). First, mesenchymal cells were depleted of functional cell surface proteoglycans by growing them in the presence of 20 mM sodium chlorate, which inhibits sulfation of cell surface glycosaminoglycans. Chlorate-treated mesenchymal cells were unable to attach to rADAM 12-cys (Fig. 3 A), but retained their ability to adhere to laminin (not shown). Thus, the lack of cell attachment on rADAM 12-cys was not due to chlorate-induced cytotoxicity. The inhibitory effect of sodium chlorate on cell attachment was restored by including 10 mM sodium sulfate in the chlorate-containing culture medium (not shown). Secondly, cell attachment was significantly inhibited when heparin or heparan sulfate was included in the assays. Similarly, cell attachment to rADAM 12-DC was inhibited by heparin (Fig. 3 B). In contrast, hyaluronic acid or chondroitin sulfate ABC did not interfere with cell adhesion (Fig. 3 A). Heparin was very potent in inhibiting cell attachment. For most of the cell lines tested, 1 $\mu\text{g}/\text{ml}$ heparin reduced cell attachment to 50%, and 10–20 $\mu\text{g}/\text{ml}$ led to a complete inhibition (not shown). The only exception was RD rhabdomyosarcoma cells, which exhibited only a partial inhibition of cell attachment when assays were performed in the presence of heparin but almost a complete inhibition (90%) when heparin was administered in the form of heparin-albumin (not shown). Thirdly, removal of cell surface heparan sulfate chains by pretreatment of the cells with heparitinase reduced cell attachment, whereas chondroitinase ABC had no effect (not shown). No effect on cell attachment was observed when cells were pretreated with $\beta 1$ integrin function-blocking mAbs (Fig. 3 A). These results demonstrate that attachment of mesenchymal cells to rADAM 12-cys and rADAM 12-DC was critically dependent on cell surface heparan sulfate.

To identify the nature of the heparan sulfate proteoglycan, which provides the heparan sulfate chains for rADAM 12-cys- and ADAM 12-DC-mediated cell adhesion, we took advantage of the ARH-77 B lymphoid cell system (Langford et al., 1998; Liu et al., 1998). The ARH-77 cell line, which was derived from a human plasma cell leukemia, expresses very little heparan sulfate on the cell surface. However, when transfected with cDNAs encoding members of the syndecan family, these ARH-77 cells express functional cell surface syndecans that bear heparan sulfate chains (Langford et al., 1998; Liu et al., 1998). A series of stably transfected cell lines that express either syndecans-1, -2, or -4, or glypican-1 on their cell surface (Liu et al., 1998) were tested in a cell adhesion assay (Fig. 4 A). As expected, the parental ARH-77 cells transfected with vector only (ARH-77 neo) did not attach to rADAM 12-cys. Cells transfected with either syndecan-1,-2, or -4 at

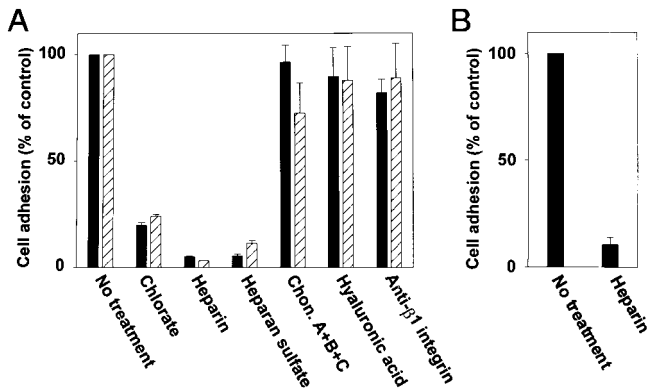


Figure 3. Cell attachment on rADAM 12-cys is dependent on cell surface heparan sulfate. (A) Cell attachment of SV-HFO cells (filled bars) and MRC-5 (hatched bars) plated on rADAM 12-cys. (B) Cell attachment of SV-HFO cells (filled bars) plated on rADAM 12-DC. The assays were performed in the presence or absence of the reagents indicated (chlorate 20 mM and the other reagents 10 μ g/ml). Ordinates represent percentage adhesion. Each point represents the mean \pm SEM of triplicate wells.

tached to rADAM 12, but did not spread, whereas glypican-1-transfected cells did not attach. Similarly, ARH-77 cells expressing syndecan-1 also attached to rADAM 12-DC (Fig. 4 B). Adhesion of syndecan-1-transfected cells to rADAM 12-cys (Fig. 4 C) or rADAM 12-DC (not shown) is blocked by compounds that interfere with the function of heparan sulfate chains. Moreover, cells expressing syndecan-1 lacking all three heparan sulfate attachment sites (ARH-77-TDM) were unable to attach to rADAM 12-cys (Fig. 4 C). Some of the syndecan-1 TDM molecules do bear chondroitin sulfate chains (Langford et al., 1998), but apparently cannot mediate cell attachment to rADAM-cys. Together these data demonstrate that adhesion of the ARH-77 cells to ADAM 12 is mediated by heparan sulfate chains of syndecan.

The finding that glypican-1-expressing cells did not adhere to rADAM 12-cys indicated that cell surface heparan sulfate chains alone were not sufficient for adhesion to rADAM 12-cys and suggested a role for the syndecan core proteins. To examine this, we analyzed adhesion to rADAM 12-cys of ARH-77 cells expressing various mutant and chimeric forms of syndecan-1, including syn/279t (syndecan-1 lacking its cytoplasmic tail), syn/glyp (a chimeric proteoglycan composed of the syndecan-1 extracellular domain and a glycosyl phosphatidylinositol (GPI) anchor for attachment to the cell surfaces), and glyp/syn (a chimeric proteoglycan composed of the glypican extracellular domain and the syndecan transmembrane and cytoplasmic domains). It has been shown previously that both the mutant and chimeric forms of syndecan are capable of binding ARH-77 cells to type I collagen and to fibronectin (Liu et al., 1998; this study; not shown). Representative syndecan-1 and glypican-1 cell surface immunostainings are shown as insets in Fig. 4 D. However, none of these mutant cell lines exhibited significant cell adhesion to rADAM 12-cys (Fig. 4 D). Taken together these results demonstrate that different syndecan family members can mediate cell adhesion to rADAM 12-cys. Moreover, in ad-

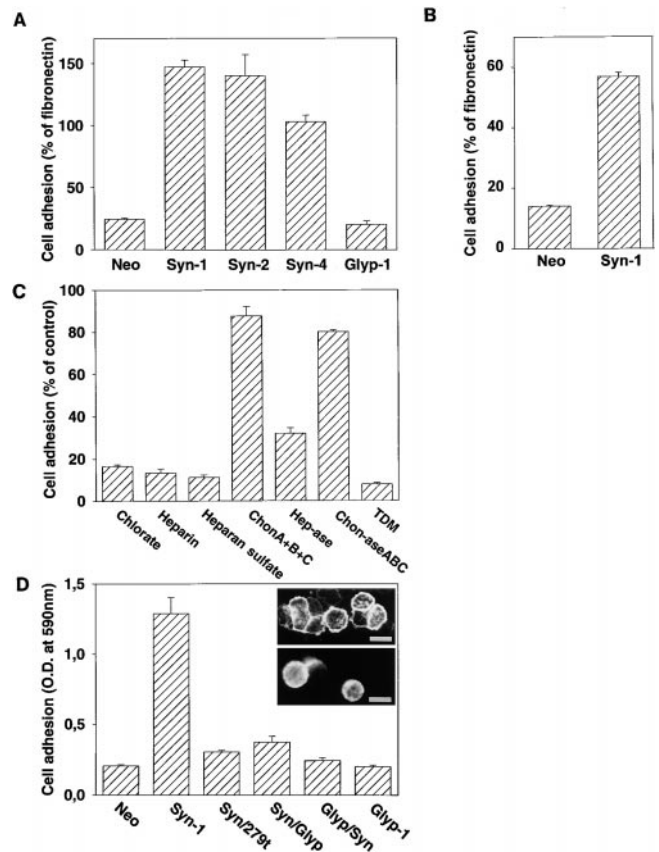


Figure 4. Cell attachment to rADAM 12-cys is mediated through the syndecans. (A) Cell attachment of ARH-77 cells transfected with either an empty vector (Neo), syndecan-1, -2, or -4 (Syn-1, -2, or -4), or with glypican-1 (Glyp-1) and plated on rADAM 12-cys (20 μ g/ μ l) or in B, on rADAM 12-DC. (C) Cell attachment on rADAM 12-cys of syndecan-1-expressing cells treated with chlorate, heparin, heparan sulfate, chondroitin sulfate ABC (Chon A+B+C), heparitinase (Hep-ase) or chondroitinase ABC (Chon-ase ABC). ARH-77-TDM cells express syndecan-1 lacking heparan sulfate attachment sites but with intact chondroitin sulfate chains. (D) Cell attachment on rADAM 12-cys of ARH-77 cells expressing truncated syndecan-1 (Syn/279t) or chimeric proteoglycans (Syn/Glyp and Glyp/Syn). Each point represents the mean \pm SEM of triplicate wells. Ordinates represent percentage of adhesion or OD values at 590 nm. Insets in D show syndecan-1 cell surface immunostaining of the ARH-77 Syn/Glyp cells (upper) and glypican-1 cell surface immunostaining of ARH-77 Glyp/Syn (lower). Bars, 22 μ m.

dition to the critical role of heparan sulfate chains, both the extracellular and cytoplasmic domains of the syndecan core protein are required for the ADAM 12-mediated cell adhesion process.

Having established the critical role of syndecans in rADAM 12-cys-mediated cell adhesion, we wanted to explore which syndecans can be functional in the mesenchymal cells. We examined RD rhabdomyosarcoma cells, which express syndecans-1 (80 kD), syndecan-2 (50 kD), and syndecan-4 (35 kD) based on RT-PCR and Western blotting (Fig. 5, A and B). Cell surface biotinylated RD cells were extracted and applied on an affinity-matrix to which rADAM 12-cys had been immobilized. Elution of bound material and subsequent gel separation and West-

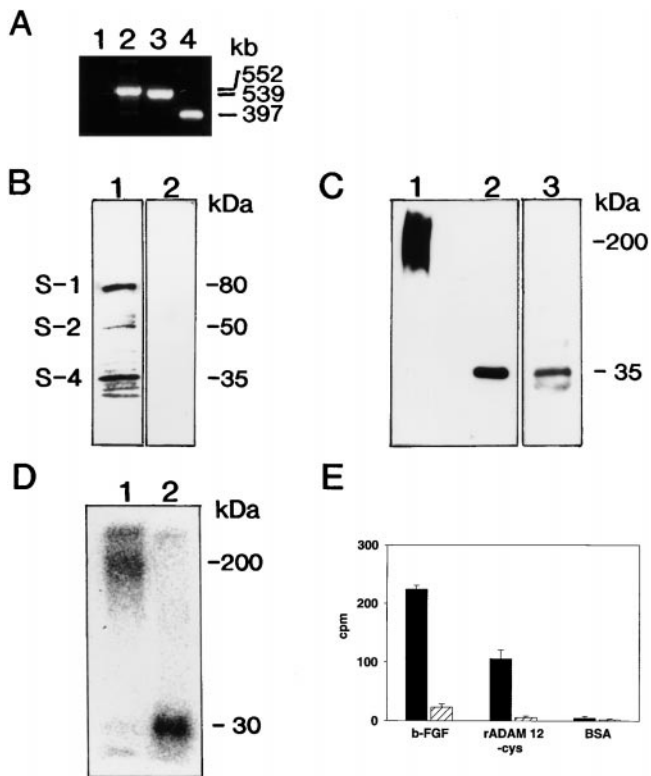


Figure 5. Syndecan-4 binds to rADAM 12-cys. (A) RT-PCR demonstrates the expression of syndecan-1 (lane 2), -2 (lane 3), and -4 (lane 4) transcripts by the RD cells. Lane 1 is a negative control. (B) RD cell extracts were separated by DEAE Sephacel, treated with heparitinase and chondroitinase resolved by SDS-PAGE, and membranes probed with the pan-syndecan antibody that recognizes the conserved C2 region in the cytoplasmic domains of all the syndecan family members (lane 1) or a pre-immune serum (lane 2). (C) SDS-PAGE of syndecan-4 from RD cells isolated by rADAM 12-cys affinity chromatography. Lane 1 shows a smeared band with an apparent M_r 200,000. After treatment with heparitinase, a single distinct band with an apparent M_r 35,000 (lane 2) is seen. A polyclonal antibody to syndecan-4 identified this band as syndecan-4 (lane 3). (D) Purified iodinated syndecan-4 before (lane 1) and after (lane 2) removal of the heparan sulfate glycosaminoglycan side chains by heparitinase treatment. (E) Solid phase binding assay demonstrating that syndecan-4 binds bFGF and rADAM 12-cys (filled bars) and that treatment of syndecan-4 with heparitinase completely inhibits its binding to rADAM 12-cys (hatched bars).

ern blotting showed a smear of an approximate M_r of 200,000 (Fig. 5 C, lane 1). After digestion, the material with heparitinase, a sharp band of 35 kD, was seen (Fig. 5 C, lane 2). Syndecan-4 has a similar molecular mass and the protein was identified in Western blotting using antibodies specific for syndecan-4 (Fig. 5 C, lane 3). These results indicate syndecan-4 as one potential adhesion receptor for rADAM 12-cys. Since solubilization of syndecans required the use of urea in the extraction buffer (Hoffman et al., 1998), we do not know whether syndecan-4 is the sole syndecan used by the RD cells when they attach to rADAM 12-cys.

The interaction between native human syndecan-4 and rADAM 12-cys was confirmed in a solid-phase binding assay (Fig. 5, D and E). As demonstrated in Fig. 5 E,

rADAM 12-cys exhibited significant syndecan-4 binding, similar to that of bFGF. Thus, when 800 cpm of 125 I-labeled human syndecan-4 (Fig. 5 D) was added in the assay (Fig. 5 E), an average of 224 cpm (\pm 7.1) bound to bFGF, 105 cpm (\pm 15.1) bound to rADAM 12-cys, and 5 cpm (\pm 3.1) to BSA. Heparitinase treatment of syndecan-4 completely destroyed its ability to bind to both rADAM 12-cys and bFGF, demonstrating that the heparan sulfate glycosaminoglycan chains were crucial for binding of these two ligands.

Full-Length Human rADAM 12-GFP Binds to Syndecan-expressing Cells But Not to Cells Lacking Cell Surface Syndecans

The results presented so far were obtained using rADAM 12-cys made in *E. coli* or rADAM 12-DC made in insect cells. To confirm the interaction of full-length ADAM 12 with cell surface syndecans, we generated a GFP-tagged form of ADAM 12-S expressed in COS-7 cells. Culture medium from these cells was collected and mixed with ARH-77 cells expressing syndecans or control ARH-77 cells that do not express syndecans. After a 1-h incubation at 4°C or at 37°C, ARH-77 cells were examined by fluorescence microscopy. Distinct punctate cell surface fluorescence was observed on the syndecan-expressing cells but not on control cells (Fig. 6, A, B, and D). No fluorescence was observed when the syndecan-expressing ARH-77 cells were pretreated with heparitinase or when heparin was included in the medium, or when the cells were incubated with medium containing GFP without ADAM 12-S (Fig. 6, C-E). These data support the model that ADAM 12 binds to cell surfaces primarily via syndecans.

Integrin $\beta 1$ Is Involved in Cell Spreading

When mesenchymal cells were treated with function-blocking mAbs to $\beta 1$ integrin (A1B2 and P5D2) the cells attached but no longer spread on rADAM 12-cys (Fig. 7, B-D). More than 90% of these cells had a rounded morphology with several fine 7–10- μ m long cytoplasmic projections without organized focal adhesions and stress fibers. In parallel experiments, these antibodies inhibited cell attachment of RKO colon carcinoma cells to laminin-1 and -10 (not shown), confirming the function-blocking activity of the preparation of purified mAbs used. As a further control, we tested a control IgG and found that this mAb had no effect on cell attachment and spreading (not shown). The data shown represent experiments done with the SV-HFO cell line and are representative of the inhibitory effect of function-blocking $\beta 1$ integrin mAbs on the spreading of the other mesenchymal cells used in this study. Finally, we examined cell spreading of SV-HFO, MRC-5, IMR-90, and RD cells that had been treated for 3 h before the experiment with 25 μ g/ml cycloheximide to inhibit protein synthesis. Such treatment had no effect on cell attachment and spreading on rADAM 12-cys (not shown), indicating that cells bind and spread on the supplied rADAM 12-cys and not to substances secreted by the cells during the assay period.

To provide further evidence that integrin $\beta 1$ is involved in rADAM 12-mediated spreading, we used genetically modified cells. Integrin $\beta 1$ -null mice are early embryonal

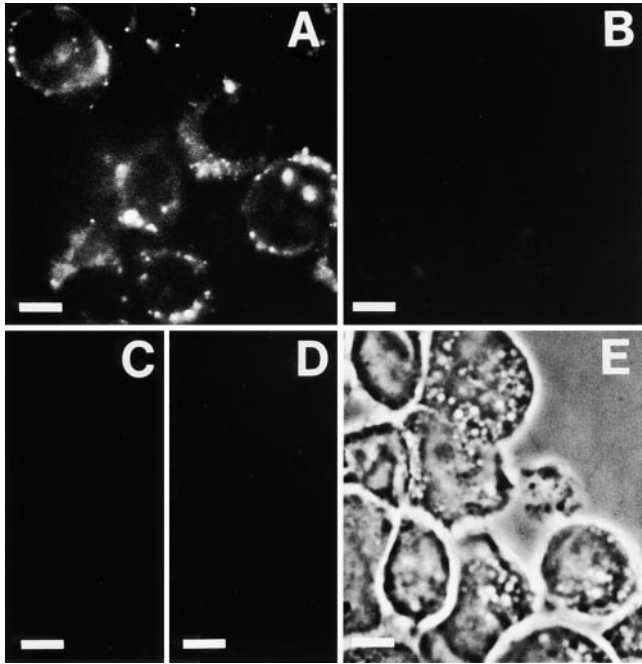


Figure 6. Full-length human ADAM 12 binds to syndecan-expressing cells. ARH-77 cells were incubated with ADAM 12-GFP-containing medium for 1 h at 4°C and examined by fluorescence microscopy. (A) Distinct cell surface fluorescence is observed along the cell surface of ARH-77 cells transfected with syndecan-1, but (B) no cell surface fluorescence is seen in control ARH-77 cells transfected with vector only. (C) Secreted GFP (not linked to ADAM 12) did not bind to syndecan-1-expressing cells, and (D) no fluorescence was observed when the cells had been pretreated with heparitinase. Phase contrast of the control ARH-77 neo cells examined in B is shown in E. Bars, 20 μ m.

lethal, precluding establishment of defined $\beta 1$ integrin^{-/-} cell populations in culture (Fässler and Meyer, 1995; Stephens et al., 1995). To overcome this problem, we established chondroblasts from conditional $\beta 1$ integrin knock-out mice (Potocnik, A.J., C. Brakebusch, and R. Fässler, manuscript submitted for publication), immortalized them with simian virus 40 large T antigen, and subsequently deleted the $\beta 1$ integrin by a cre recombinase transfection as described in Materials and Methods.

Chondroblast^{+/+} cells attached, and >90% of the cells spread on rADAM 12-cys and laminin-1, respectively (Fig. 8, A and B). Chondroblast^{-/-} cells attached on rADAM 12-cys, but their spreading was significantly reduced (Fig. 8 C). As expected, $\beta 1$ integrin-null chondroblasts did not attach on laminin-1 (Fig. 8 D). Moreover, we confirmed that both chondroblast^{+/+} and ^{-/-} cell attachment to rADAM 12-cys was completely inhibited by heparin (Fig. 8 E). The lack of detectable $\beta 1$ integrin expression on the surface of ^{-/-} chondroblasts was confirmed by FACS analysis (Fig. 8 F).

Multiple Integrin α Subunits Are Involved in Cell Spreading on rADAM 12-cys

To determine which integrin α chain(s) were involved in cell spreading, we used a series of function-blocking mAbs in the attachment assays. No effect on cell attachment or cell spreading was observed with function-blocking antibodies to integrin $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$ when added individually (not shown). When administered in pairs of two mAbs, integrin $\alpha 3$ and $\alpha 5$, $\alpha 3$ and $\alpha 6$, and $\alpha 5$ and $\alpha 6$ mAbs had no effect on cell attachment, but a partial (~30%) inhibitory effect on cell spreading (not shown). Other combinations were without effect on cell spreading. Finally, when integrin $\alpha 3$, $\alpha 5$, and $\alpha 6$ mAbs were combined, 90% inhibition of cell spreading was seen (Fig. 9). These results suggest that multiple integrin α subunits participate in cell spreading on rADAM 12-cys.

Syndecan- $\beta 1$ Integrin Cross-talk Is Nonfunctional in Carcinoma Cells

Our data suggested that mesenchymal cells use syndecans as the primary cell attachment receptor to rADAM 12-cys, and use $\beta 1$ integrin to mediate cell spreading. However, in a previous report, we found that carcinoma cells had a rounded morphology and did not spread upon attaching to rADAM 12-cys (Iba et al., 1999). We therefore tested whether cell spreading could be induced (Fig. 10). Addition of either Mn²⁺ or $\beta 1$ integrin-activating antibodies changes the conformation of the extracellular domain of $\beta 1$ integrins to an active ligand-binding state by increasing the apparent affinity (Hynes, 1992; Humphries, 1996). Carcinoma cells were plated on laminin-1, fibronectin, rADAM 12-cys, or rADAM 12-DC for 30 min, followed

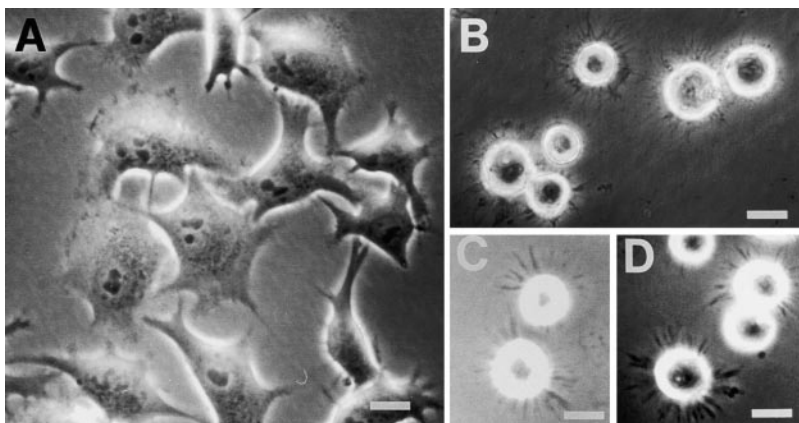


Figure 7. Cell spreading in response to rADAM 12-cys is mediated by $\beta 1$ integrin. (A) Cell attachment of SV-HFO cells with the characteristic spread morphology is shown. (B–D) In the presence of function-blocking mAbs to $\beta 1$ integrin (AIIB2), the cells attached but no longer spread, and displayed a rounded morphology with many cytoplasmic projections. Three representative fields are shown. Bars: 10 μ m (A, B, and D); 12 μ m (C).

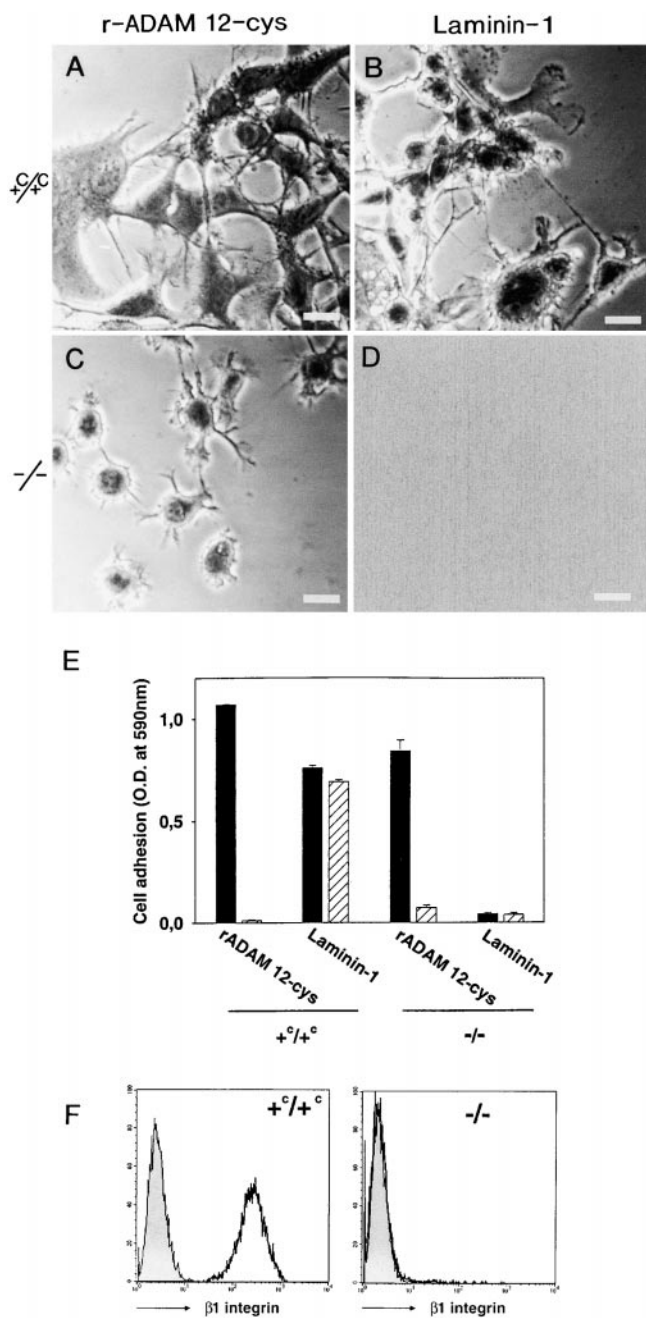


Figure 8. $\beta 1$ integrin-deficient chondroblasts attach but do not spread on rADAM 12-cys. (A and B) Cell attachment of $\beta 1$ integrin chondroblasts ($+c/+c$) plated on rADAM 12-cys and laminin-1, respectively. (C and D) Cell attachment of $\beta 1$ integrin $-/-$ chondroblasts on rADAM 12-cys and laminin-1, respectively. Bars, 14 μm . (E) Chondroblast cell attachment (filled bars) and the effect of 10 $\mu\text{g/ml}$ of heparin (hatched bars) on rADAM 12-cys or laminin-1 as a substrate. The substrate concentration was 20 $\mu\text{g/ml}$. Ordinate represents OD values at 590 nm. (F) FACS analysis confirming the absence of $\beta 1$ integrin on $-/-$ chondroblasts.

by another 30-min incubation in the presence or absence of Mn^{2+} . Carcinoma cells adhered tightly on laminin-1 and fibronectin and were well-spread. The addition of Mn^{2+} or $\beta 1$ integrin-activating antibodies neither increased the number of adherent cells nor the extent of spreading (Fig.

10, A, B, and I). In contrast, when cells were plated on rADAM 12-cys or rADAM 12-DC they adhered well but were not spread (Fig. 10, C and G). Addition of 1 mM Mn^{2+} or $\beta 1$ integrin-activating antibodies induced immediate spreading (Fig. 10, D, F, and H), which was similar to cells plated on laminin or fibronectin. No effect was seen when the control K20 $\beta 1$ integrin mAb was added (Fig. 10 E).

Discussion

The data obtained in this study demonstrate that the rADAM 12-cys and rADAM 12-DC serve as potent adhesion substrates for a variety of mesenchymal cells, including lines of fibroblastic, osteoblastic, and myoblastic cell origin. Furthermore, rADAM 12-GFP binds to the cell surface. In the adhesion process, syndecan and integrin cell surface receptors act cooperatively to generate signals for cell adhesion and cell spreading, and for the assembly of focal adhesions and actin stress fibers. Syndecans are involved in cell attachment and $\beta 1$ integrins in the subsequent cell spreading. Interestingly, carcinoma cells attach but fail to spread on rADAM 12-cys and rADAM 12-DC. Their spreading, however, can be efficiently induced by addition of either 1 mM Mn^{2+} or the $\beta 1$ integrin-activating mAb 12G10. These data support a model in which the binding of cell surface syndecans with the ADAM 12 cysteine-rich domain leads to an integrin-dependent spreading of cells. Interestingly, in carcinoma cells, the binding to rADAM 12-cys by syndecans does not allow the integrin-mediated cell spreading. The signaling machinery involved in this syndecan-integrin cross-talk is unknown.

Syndecans are abundant cell surface heparan sulfate proteoglycans involved in multiple biological processes by virtue of the binding of their glycosaminoglycans to growth factors, extracellular matrix proteins, cell adhesion molecules, and proteases and protease inhibitors (Carey, 1997; Rapraeger and Ott, 1998; Woods et al., 1998; Bernfield et al., 1999; Zimmermann and David, 1999). It is calculated that heparan sulfate proteoglycans occupy $>25\%$ of the cell surface of many cells (Lander, 1999). The heparan sulfate chains, which are attached to the ectodomain of the core protein, have a strong negative charge and interact with proteins by forming multiple electrostatic contacts with basic amino acids. This interaction usually leads to relatively low affinity binding (dissociation constants in the range of $2 \times 10^{-8} - 2 \times 10^{-5}$ M) (Lander, 1998). In this study, low concentrations of heparin (10–20 $\mu\text{g/ml}$) completely blocked the interaction of mesenchymal cells with immobilized rADAM 12-cys and rADAM 12-DC, and pretreatment of the cells with heparitinase likewise inhibited the interaction. Moreover, if cells were incubated with chlorate, an inhibitor of sulfation, no adhesion was observed. Finally, cells that do not contain cell surface heparan sulfates do not attach to rADAM 12-cys. This was shown with ARH-77 cells that express very little cell surface heparan sulfate and with several ARH-77 transfectants used as a model system to examine which cell surface heparan proteoglycan supports rADAM 12-cys-mediated cell adhesion. ARH-77 cell lines transfected with either syndecan-1, -2, or -4 attached to rADAM 12-cys, whereas cells transfected with glypican-1 did not, demonstrating

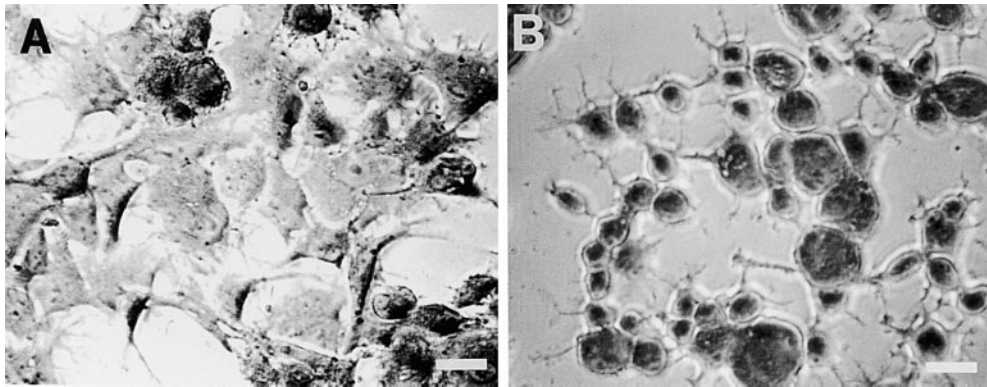


Figure 9. Multiple integrins are involved in rADAM 12-cys-mediated cell spreading. (A) MRC-5 cells plated on rADAM 12-cys had a spread and flattened morphology. (B) In contrast, cells treated with function-blocking mAbs to $\alpha 3 + \alpha 5 + \alpha 6$ integrins attached but did not spread. Note the rounded cell morphology with numerous cytoplasmic projections. Bars: 22 μm (A); and 26 μm (B).

that different syndecan members are able to mediate cell adhesion to rADAM 12-cys.

The syndecan family consists of four members, designated syndecans 1–4. Each syndecan shows a specific and distinct expression pattern during development and in adult tissue (Carey, 1997; Rapraeger and Ott, 1998; Woods et al., 1998; Bernfield et al., 1999; Zimmermann and David, 1999). Syndecan-1 is primarily expressed on epithelial cells, syndecan-2 (fibroglycan) on fibroblastic, endothelial, and liver cells, and syndecan-3 (N-syndecan) on neuronal cells. Syndecan-4 (ryudocan, amphiglycan) shows a widespread expression pattern. In addition, syndecan-4 is specifically concentrated into focal adhesions with integrins, and may play an important role in the control of focal adhesion formation and the organization of the actin cytoskeleton (Woods and Couchman, 1994; Baciou and Goetinck, 1995; Echtermeyer et al., 1999; Longley et al., 1999). However, many cells express more than one syndecan (Kim et al., 1994). To test the idea that one syndecan might be preferentially used by specific cell types in the process of cell adhesion to rADAM 12-cys, we isolated proteins from biotinylated RD cell surfaces that bind to rADAM 12-cys by affinity chromatography. Indeed, syndecan-4 with a core protein of ~ 35 kD bound detectably to immobilized rADAM 12-cys. A solid phase binding assay devised previously by Kojima et al. (1996) confirmed the interaction of purified human syndecan-4 with rADAM 12-cys. Thus, syndecan-4 represents a potentially important binding partner for ADAM 12 in the cell adhesion response described here and possibly also in modulating cell behavior in myogenesis (Yagami-Hiromasa et al., 1995; Gilpin et al., 1998) and cancer (Iba et al., 1999).

Syndecans often serve as coreceptors. The classical example is that syndecans supply the glycosaminoglycans necessary for bFGF to bind to its high affinity receptor (Rapraeger et al., 1991). More recently, several reports have pointed to a role for syndecans, in particular syndecan-4, as coreceptors for integrins (Woods et al., 1986; Saoncella et al., 1999). We also show in our study that both syndecans and integrins are involved in rADAM 12-cys-mediated cell attachment and spreading. The initial cell attachment is mediated by syndecans, and subsequent cell spreading by functional $\beta 1$ integrins. This conclusion is based on the observation that cells treated with function-blocking antibodies to integrin $\beta 1$ and cells in which the $\beta 1$ integrin gene was deleted attach to rADAM 12-cys but do

not spread. Mesenchymal cells express several $\beta 1$ integrins. For example, SV-HFO osteoblastic cells express $\beta 1$, $\alpha 1$ – $\alpha 3$ and $\alpha 5$ – $\alpha 6$, and $\alpha v\beta 3$ (Iba et al., 1996). A combination of function-blocking antibodies reacting with $\alpha 3$, $\alpha 5$, and $\alpha 6$ integrins inhibited cell spreading. Whereas treatment of cells with antibodies in pairs showed partial inhibition of spreading, single antibody treatment had no effect. These data clearly suggest that syndecan and integrins cooperate during cell adhesion and spreading.

Integrins have been identified previously as receptors for the ADAMs and for the closely related snake venom metalloproteases (SVMPs). Several SVMPs interact with $\alpha \text{IIb}\beta 3$ and $\alpha 2\beta 1$ integrins, and through this binding compete with their natural ligands, causing severe hemorrhage in the bite victim (Jia et al., 1996). In the mouse, ADAM 2 (fertilin β) on the sperm cell surface interacts through the disintegrin-like domain with $\alpha 6\beta 1$ integrin on eggs in the fertilization process (Almeida et al., 1995; Chen et al., 1999a,b). The importance of ADAM 2 in this process is also supported by the finding that mice deficient of ADAM 2 are infertile (Cho et al., 1998). Further evidence for a role of the disintegrin-like domain in cellular interactions was provided by Zhang et al. (1998) and Nath et al. (1999). Thus, the Arg-Gly-Asp (RGD)-containing disintegrin-like domain of ADAM 15 made in *E. coli* interacts with the $\alpha v\beta 3$ integrin (Zhang et al., 1998). ADAM 15 made in COS cells as a chimeric protein containing the extracellular domain of ADAM 15 fused to the Fc portion of human IgG interacts with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins on different hemopoietic cells. We have demonstrated that rADAM 12-cys and rADAM 12-DC support cell adhesion (Iba et al., 1999; Zolkiewska, 1999; this study). Furthermore, we show here that full-length ADAM 12 (rADAM 12-GFP) binds to cell surface syndecans. Based on these results, it appears that the cysteine-rich domain of ADAM 12 comprises most of the syndecan-mediated cell binding activity. Interestingly, the cysteine-rich domain of the hemorrhagic SVMP atrolysin A has been recently demonstrated to inhibit collagen-stimulated platelet aggregation, indicative of an interaction with integrin $\alpha 2\beta 1$ (Jia et al., 2000). Thus, for ADAM 12, and possibly for other ADAMs and SVMPs, the cysteine-rich domain is involved in mediating cellular interactions via syndecans and integrins.

Integrins regulate many cellular events, including shape, adhesion, migration, and survival of cells. The execution of these functions depends on the activation state of inte-

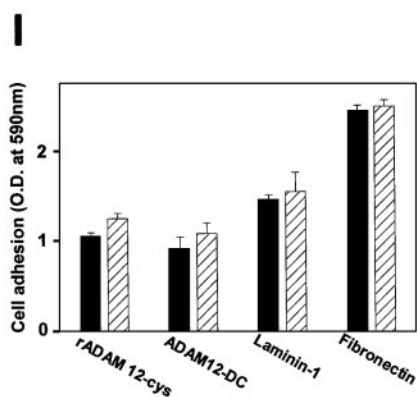
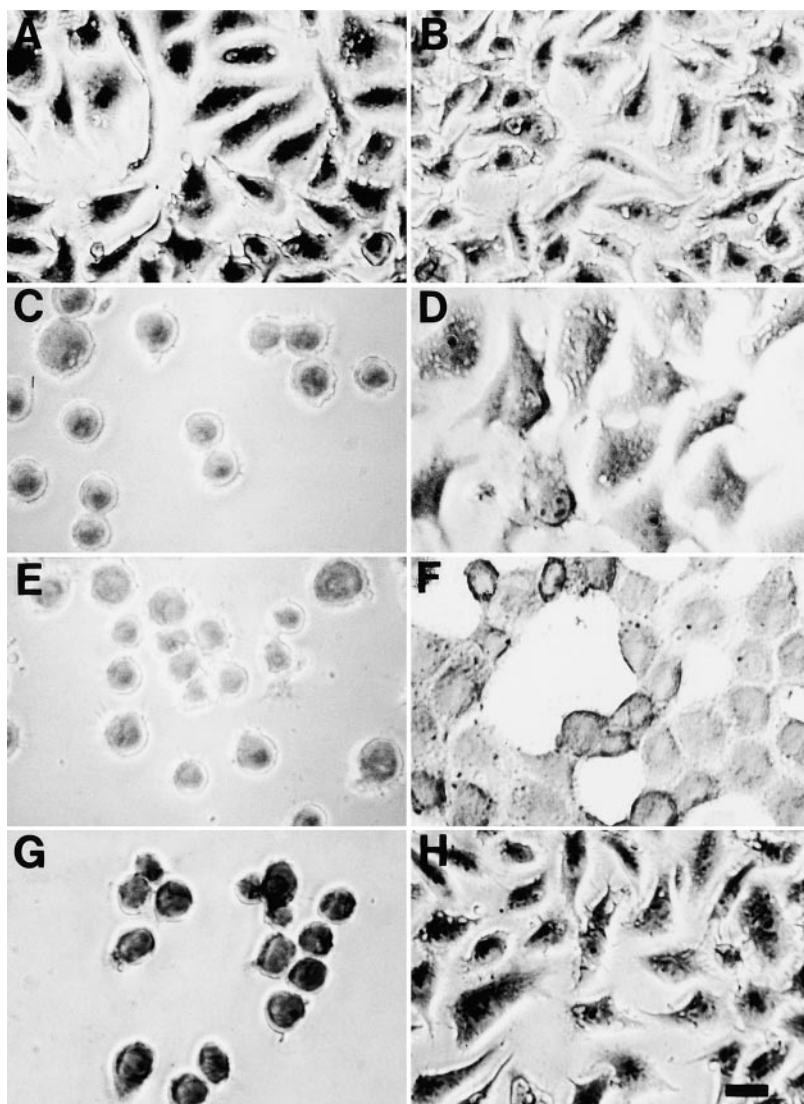


Figure 10. Carcinoma cells can be induced to spread by activating the $\beta 1$ integrins. RKO colon carcinoma cells were allowed to attach to laminin (A and B), rADAM 12-cys (C–F), or rADAM 12-DC (G and H) for 30 min followed by the addition of reagents known to stimulate $\beta 1$ integrin activity. RKO cells were plated on laminin either in the absence (A) or in the presence (B) of 1 mM Mn^{2+} . RKO cells were plated on rADAM 12-cys in the absence (C), or presence (D) of 1 mM Mn^{2+} , or in the presence of control $\beta 1$ integrin mAb K20 (10 $\mu g/ml$) (E), or the $\beta 1$ integrin-activating mAb 12G10 (10 $\mu g/ml$) (F). Similarly RKO cells were plated on rADAM 12-DC in the absence (G) or presence (H) of 1 mM Mn^{2+} . Bar, 12 μm . (I) RKO cell attachment (filled bars) and the effect of 1 mM Mn^{2+} (hatched bars) on rADAM 12-cys, rADAM 12-DC, laminin-1, or fibronectin as substrates (20 $\mu g/ml$). Ordinate represents OD values at 590 nm.

grins. In recent years, several laboratories have shown that integrins can be expressed on the cell surface but are unable to recognize and bind their ligands. Certain signals such as the activation of R-ras (Zhang et al., 1996) and the phosphorylation of integrin cytoplasmic domain-associated protein-1 (ICAP-1) (Bouvard and Block, 1998) or the focal adhesion protein VASP (vasodilator-stimulated phosphoprotein) (Aszodi et al., 1999) may change the conformation of integrins and thereby modulate the ability to

bind ligands. These changes are referred to as inside-out signaling. The data presented in this paper suggest a model in which the initial binding of ADAM 12 cysteine-rich domain by syndecan on mesenchymal cells triggers a conformational change of the ADAM 12-cys, which may allow integrins to bind ADAM 12-cys and subsequently the cells to spread. This hypothesis is supported by several findings. First, blocking antibodies to integrins inhibit spreading of mesenchymal cells on rADAM 12-cys. Secondly, chondro-

blasts which lack the expression of $\beta 1$ integrin fail to spread on rADAM 12-cys. Thirdly, carcinoma cells can tightly bind to rADAM 12-cys, but in contrast to mesenchymal cells, are unable to spread on rADAM 12-cys. The inability to spread can be efficiently overcome by either adding activating antibodies or Mn^{2+} , which both can change the conformation of the integrins and allow integrin binding. The inability of carcinoma cells to spread on rADAM 12-cys adds another twist to our findings. It suggests that in carcinoma cells, the binding of rADAM 12-cys to syndecan may lead to an inactivation of integrins through syndecan-mediated signals that consequently does not allow the integrins to bind to rADAM 12-cys. Alternatively, the binding of rADAM 12-cys to syndecan may fail to signal positively to the integrins in the carcinoma cells. Several questions need to be addressed in the future. It will be interesting to see whether the cross-talk between syndecans and integrins is only operating in transformed carcinoma cells or also in primary epithelial cells. Furthermore, we do not know why syndecan signaling to integrins is different between mesenchymal cells and carcinoma cells. Work addressing these interesting questions is currently in progress in our laboratory.

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