Published in final edited form as:

Exp Cell Res. 2011 July 15; 317(12): 1774–1784. doi:10.1016/j.yexcr.2011.04.005.

Short form of $\alpha 9$ promotes $\alpha 9\beta 1$ integrin-dependent cell adhesion by modulating the function of the full-length $\alpha 9$ subunit

Shigeyuki Kon[†], Amha Atakilit[†], and Dean Sheppard^{†,*}

[†]Lung Biology Center, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143

Abstract

The $\alpha 9\beta 1$ integrin is a multifunctional receptor that interacts with a variety of ligands including vascular cell adhesion molecule 1, tenascin-C and osteopontin. A 2.3-kb truncated form of $\alpha 9$ integrin subunit cDNA was identified by searching the Medline database. This splice variant, which we called the short form of $\alpha 9$ integrin (SF $\alpha 9$), encodes a 632-aa isoform lacking transmembrane and cytoplasmic domains, and its authentic expression was verified by PCR and western blotting. SF $\alpha 9$ is expressed on the cell surface, but cannot bind ligand in the absence of the full-length $\alpha 9$ subunit. Over-expression of SF $\alpha 9$ in cells expressing full-length $\alpha 9$ promotes $\alpha 9$ -dependent cell adhesion. This promoting effect of SF $\alpha 9$ requires the authentic cytoplasmic domain of the co-expressed full-length $\alpha 9$ subunit. Thus, SF $\alpha 9$ is a novel functional modulator of $\alpha 9\beta 1$ integrin by inside-out signaling.

Keywords

SF $\alpha 9$; $\alpha 9\beta 1$ integrin; cell adhesion

Introduction

Integrins are a family of heterodimeric type I transmembrane proteins composed of an α subunit and a β subunit, which are non-covalently bound [1]. At the present time, 18 different α subunits and 8 different β subunits are known, which give rise to 24 distinct $\alpha\beta$ heterodimer [2]. They are involved in a wide range of cellular processes including cell adhesion, migration, differentiation, proliferation, apoptosis, and cancer metastasis by recognizing diverse ligands in the extracellular matrix or on the cell surface. Ligand binding is mediated by the N-terminal portions of each integrin subunit, containing seven FG-GAP repeats termed a β -propeller domain [2, 3]. The avidity modulation of integrins occurs in two directions, signaling from the extracellular microenvironment into the cell known as "outside-in signaling", and from intracellular to extracellular domains of integrins known as "inside-out signaling" [2, 4, 5].

© 2010 Elsevier Inc. All rights reserved.

*Corresponding author. UCSF Mission Bay, Rock Hall, Room 548E, 1550 4th Street, San Francisco, CA 94158-2324, USA., TEL: +1-415-514-4269. FAX: +1-415-514-4278., deans@itsa.ucsf.edu (Dean Sheppard).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The $\alpha 9\beta 1$ integrin is widely expressed and binds to a variety of ligands, including tenascin-C, osteopontin, VCAM-1, VEGF-A, -C, -D, and several members of the ADAM family [6–10]. At least two proteins that directly interact with the $\alpha 9$ integrin subunit cytoplasmic domain have been reported to modulate $\alpha 9\beta 1$ -mediated cell migration and cell spreading. SSAT (spermidine/spermin *N*¹-acetyltransferase) binds to the integrin $\alpha 9$ cytoplasmic domain and modulates inward rectification of the Kir4.2 (inward-rectifier K⁺) channel to enhance $\alpha 9$ integrin-dependent cell migration [11, 12]. Paxillin binding to the integrin $\alpha 9$ cytoplasmic domain mediates inhibition of cell spreading by $\alpha 9\beta 1$ [13, 14]. However no molecule has yet been identified as a specific modifier of integrin $\alpha 9$ -dependent adhesion.

We found a sequence predicted to encode an alternative splice variant of the $\alpha 9$ integrin subunit on the NCBI Database. This cDNA encodes a truncated 632-aa protein, which has β -propeller domain, thigh domain, and a novel 19-aa sequence at its C-terminus. We named this isoform the short form of $\alpha 9$ (SF $\alpha 9$) and found that the predicted protein is endogenously made and expressed on the cell surface. Although expression by itself does not mediate cell adhesion to known $\alpha 9\beta 1$ ligands, SF $\alpha 9$ enhances adhesion mediated by full-length $\alpha 9$ integrin, an effect that depends on the presence of sequences in the cytoplasmic domain of the full-length $\alpha 9$ integrin.

Materials and Methods

Cell culture and reagents

CHO cells, MEF cells, SW480 cells, human Phoenix-E (ϕ E) cells (from Gary Nolan, Stanford University, Stanford, CA) [15], A549 cells (derived from human lung adenocarcinoma), LN-229 cells (derived from human glioblastoma), G361 cells (derived from human melanoma), RD cells (derived from a human rhabdomyosarcoma), MDA-MB-435s cells (derived from human melanoma cells, although originally described as of breast cancer origin) (American Type Culture Collection) were cultured in DMEM containing 10% FBS (HyClone). Anti- $\alpha 9$ integrin antibodies Y9A2 [16], A9A1 [7], or B9A1 for flow cytometry or immunoprecipitation were generated and characterized in our laboratory. Anti- $\alpha 9$ integrin monoclonal antibody NC7 for western blotting was generated by immunizing mice with a peptide derived from the C-terminal domain of mouse $\alpha 9$, EAEKNRKENEDGWDWVQKNQ. Anti-FLAG antibody M2, and anti-Myc antibody 9E10, were obtained from Sigma-Aldrich and Roche Applied Science, respectively. Anti- $\beta 1$ integrin antibody (AB1952) was obtained from Chemicon. Anti-mouse osteopontin antibody (O-17) and anti- β -actin antibody (ab8226) used as loading controls were obtained from IBL and Abcam, respectively. The mutant form of the third fibronectin type III repeat in tenascin-C (TNfn3RAA) has been described [17, 18]. Human plasma fibronectin was obtained from Sigma-Aldrich.

SF $\alpha 9$ cDNA cloning

The cDNAs for the alternative splicing variant of $\alpha 9$ integrin (SF $\alpha 9$), FLAG-tagged SF $\alpha 9$ and Myc-tagged SF $\alpha 9$ cDNA were PCR amplified from G361 human melanoma cells using the following primers; 5'-CGGTACACCTACCTGGGCTA-3' (sense) and 5'-AGGAGTCGACAAAGGGAGACCCAGAGAGGA-3' (antisense for SF $\alpha 9$), 5'-CCGTCGTCGACCTATTTATCGTCATCATCTTTGTAGTCTTTCTGCTCTGTGCAGCTGCC-3' (antisense for FLAG-tagged SF $\alpha 9$), and 5'-CCGTCGTCGACTTACAGGTCTTCCTCAGAGATGAGTTTCTGCTCTGTGCAGCTGCC-3' (antisense for Myc-tagged SF $\alpha 9$). PCR products were digested with BglII and SalI, then inserted into BglII and SalI-digested $\alpha 9$ integrin expressing vector, $\alpha 9$ -pBabepuro. A fragment digested with BglII and SalI from Myc-tagged SF $\alpha 9$ -pBabepuro was inserted into BglII and SalI-digested $\alpha 9$ integrin expressing vector, $\alpha 9$ -pWZLblast.

Generation of stable cell lines

MEF cells expressing $\alpha 9$ integrin subunit or SF $\alpha 9$ were generated as follows. $\alpha 9$ -pBabepuro, or SF $\alpha 9$ -pBabepuro were transfected into ϕ E packaging cells by Lipofectamine2000 (Invitrogen). 3 days after transfection, virus-containing supernatants were harvested and filtered through a 0.45- μ m filter and then added to 50% confluent MEF cells in the presence of 8 μ g/ml polybrene and cultured for 18–20 h. The virus-containing medium was removed and the cells were cultured in 10% FCS DMEM supplemented with 10 μ g/ml puromycin (Sigma-Aldrich). For generation of MEF cells co-expressing $\alpha 9$ integrin subunit and SF $\alpha 9$, retroviruses were generated by transfecting $\alpha 9$ -pWZLblast, then added to SF $\alpha 9$ /MEF, SF $\alpha 9$ -Myc/MEF, or SF $\alpha 9$ -FLAG/MEF cells. MEF cells transduced by this virus were cultured in 10% FCS DMEM supplemented with 10 μ g/ml puromycin and 10 μ g/ml Blasticidin (Invitrogen). MEF cells expressing the $\alpha 9$ integrin subunit and/or SF $\alpha 9$ were identified by flow cytometry with the anti- $\alpha 9$ antibody Y9A2, and western blotting with anti- $\alpha 9$ antibody (NC7 or AF3827 from R&D Systems), anti-Myc antibody and anti-FLAG antibody. $\alpha 9$ -, $\alpha 9\alpha 5$, $\alpha 9\alpha 4$ integrin-expressing CHO cells, and $\alpha 9$ -expressing SW480 cells were previously generated in our lab [13] [17]. FLAG-tagged SF $\alpha 9$ was transfected into CHO cells or $\alpha 9$ -expressing SW480 cells. Stable clones were obtained by limiting dilution and screened with flow cytometry and western blotting.

Cell adhesion assay

The 96-well plates were coated with a mutant fragment of tenascin-C (TNfn3RAA) or plasma fibronectin overnight at 4°C, followed by blocking with 0.5% BSA in PBS for 1 hr at room temperature. Cells were suspended in DMEM containing 0.25% BSA and 200 μ l of cell suspension (at a cell density of 5×10^4 cells/well) was applied to 96-well plates and incubated for 1 hr at 37°C. The medium was removed and all wells were washed twice. Adherent cells were fixed and stained by 0.5% crystal violet in 20% methanol for 30 min. Wells were rinsed three times with water, and adherent cells were then lysed with 20% acetic acid. The resulting supernatants from each well were analyzed by an immunoreader (Bio-Rad Laboratories), and the absorbance at 595 nm was measured to determine the relative number of adherent cells.

Immunoprecipitation and western blot analysis

CHO cells, SW480 cells co-expressing $\alpha 9$ integrin and SF $\alpha 9$ or various tumor cell lines were lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and 1 $\times$ protease inhibitors (1 $\times$ Complete mini protease inhibitor cocktail, Roche Molecular Biochemicals)). Lysates were clarified by centrifugation at 16,000 *g* for 10 min at 4°C, and were then incubated with protein G-Sepharose coated with anti- $\alpha 9$ antibody NC7, anti-FLAG antibody (for CHO cells), or anti- $\alpha 9$ antibody Y9A2, A9A1, or B9A1 (for SW480 or various tumor cell lines) at 4°C 1hr. The beads were washed with the same buffer five times, and precipitated polypeptides were extracted in Laemmli sample buffer, separated by SDS-PAGE under reducing conditions, probed with NC7, anti-FLAG antibody or anti-integrin $\beta 1$ antibody (for CHO cells) or anti- $\alpha 9$ integrin (R&D) antibody (for SW480 or tumor cell lines), and detected by Plus-ECL (PerkinElmer).

Cell surface protein biotinylation

Labeling of surface proteins was performed using the lysine-directed, membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin (Thermo Scientific). Cells were washed twice with PBS at 4°C and incubated with 1.2 mg/ml sulfo-NHS-SS-biotin in PBS for 30 min at 4°C. After the sulfo-NHS-SS-biotin incubation, quenching solution was added to quench the reaction. The quenching buffer was removed, a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and 1 $\times$ protease inhibitors) was applied, and the

mixture was rotated for 30 min at 4°C. Supernatants were cleared of insoluble material by pelleting at 15,000 rpm for 10 min at 4°C. ImmunoPure-immobilized streptavidin beads (Thermo Scientific) was added to supernatant, and gently mixed for 1 h at room temperature. Streptavidin beads were then washed four times with lysis buffer and the biotinylated proteins were eluted from the beads with lysis buffer containing 50 mM DTT. Eluted samples were used for western blotting.

Flow cytometry

For GFP expression, cells were trypsinized, washed, resuspended in 100 µl of PBS, and analyzed with a FACScan flow cytometer (Becton Dickinson). For α9 integrin or SFα9 expression, cells were blocked by normal goat serum, then sequentially incubated with Y9A2 and phycoerythrin-labeled goat anti-mouse antibody before analysis.

SFα9 small hairpin RNA (shRNA) cloning, lentivirus generation, and transduction

The lentiviral vectors pSicoR, as well as the methods of shRNA cloning and lentiviral generation, were used as described [19]. Two shRNAs were designed against SFα9 using pSicoOligomaker software from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). The target sequences of two shRNA are as follows; shRNA1 target sequence: GAACCTTAAAGCTCATACT, shRNA2 target sequence: GCTGCACAGAGCAGAAATA. Oligonucleotides were ligated into the HpaI/XhoI-digested pSicoR plasmid and used to generate lentivirus as described [19].

Analysis of mRNA expression

Total RNA from various tumor cell lines was extracted by RNeasy (Qiagen). The following primers were used for RT-PCR and real-time PCR; 5'-ACCACAGTCCATGCCATCAC-3' (sense for G3PDH) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense for G3PDH), 5'-GGGTCAGGTCACAGAGAAGC-3' (sense for α9 integrin) and 5'-ACAGTCCTCTGAACGGCAAT-3' (antisense for α9 integrin), and 5'-GGGTCAGGTCACAGAGAAGC-3' (sense for SFα9) and 5'-CCACCTTGGGTGCTGAGTAT-3' (antisense for SFα9). Real-time PCR was performed by ABI Prism 7700 (Applied Biosystems). Amplified cDNA was detected with SYBR Green (Invitrogen) and standardized to ROX dye levels. cDNA concentrations were expressed as the number of cycles to threshold (Ct). Ct numbers were normalized to GAPDH in the same samples. The absolute copy numbers of particular transcripts in RD cells were calculated from standard curves generated with a 10-fold dilution series of precisely quantified cloned template DNA.

Statistical analysis

Data are presented as means ± SEM and are representative of at least three independent experiments. The statistical significance of differences between groups was calculated with a 2-tailed Student's t test. Differences were considered to be significant when $P < 0.05$ (*) or 0.005 (**).

Results

SFα9 is an alternative splicing valiant of α9 integrin

A potential splice variant of the α9 integrin subunit is shown on NCBI (Genbank ID: BC030198). This variant would encode a truncated isoform containing only the β-propeller domain and thigh domain (Fig.1A). The truncation occurs after Gln 613, which the last amino acid of the predicted thigh domain. The truncated α9 integrin cDNA contains an additional 19 amino acids present in an intron in full-length α9 integrin and a stop codon

after exon 15 (we named this region as Ex15b) at its 3' end of the mRNA (Fig.1B). We named this variant short form of $\alpha 9$ (SF $\alpha 9$).

Expression of authentic SF $\alpha 9$ mRNA in various cancer cell lines

Full-length $\alpha 9$ integrin is expressed in various tumor cell lines including LN-229 cells [20], G361 [21], and RD cells [17]. Using these cell lines and 3 additional cell lines in which full-length $\alpha 9$ mRNA is expressed, RT-PCR was performed to see the expression of authentic SF $\alpha 9$ mRNA. We found that SF $\alpha 9$ mRNA is detected in LN-229, G361, RD, and MDA-MB-435s cells (Fig.2A). The mRNA expression levels of full-length $\alpha 9$ integrin or SF $\alpha 9$ in these cell lines were analyzed by real-time PCR. Abundant full-length $\alpha 9$ integrin and SF $\alpha 9$ mRNA were expressed in RD cells as detected by RT-PCR (Fig.2B). To compare the ratio between full-length $\alpha 9$ integrin and SF $\alpha 9$ mRNA in RD cells, absolute copy number was calculated. The absolute copy number/ μ g of total RNA of full-length $\alpha 9$ integrin or SF $\alpha 9$ is $1,991,000 \pm 245,000$ or $247,000 \pm 37,300$ (Fig.2C). Thus, at least 8 times more mRNA of $\alpha 9$ integrin is expressed than SF $\alpha 9$ in RD cells. Next, we used flow cytometry to analyze surface expression of $\alpha 9$ integrin on various tumor cells using antibody Y9A2 against the extracellular domain of $\alpha 9$ integrin. We found that LN-229, G361, RD, or MDA-MB-435s, but not A549 cells, express $\alpha 9$ integrin on the cell surface. To confirm whether $\alpha 9$ integrin on tumor cell surface is functionally active, cell adhesion assays were performed using the $\alpha 9\beta 1$ specific substrate, TNfn3RAA, a recombinant form of the third fibronectin type III repeat in chicken tenascin-C mutated from the normal RGD sequence to RAA to prevent RGD-dependent interaction with RGD-recognizing integrins. Surprisingly, only LN-229, G361, or RD cells but not A549 or MDA-MB-435s adhered to TNfn3RAA (Fig.2E). RD cells adhered strongly to TNfn3RAA, whereas LN-229 or G361 showed weak-binding.

SF $\alpha 9$ is expressed on the cell surface and, by itself does not mediate adhesion to tenascin-C

Since SF $\alpha 9$ is truncated in the extracellular domain of $\alpha 9$ integrin, we first hypothesized that SF $\alpha 9$ might be a soluble protein. Cell lysate and culture medium from MEF cells expressing SF $\alpha 9$ (SF $\alpha 9$ /MEF), Myc-tagged SF $\alpha 9$ (SF $\alpha 9$ -Myc/MEF), FLAG-tagged SF $\alpha 9$ (SF $\alpha 9$ -FLAG/MEF), full-length $\alpha 9$ ($\alpha 9$ /MEF), or transfected with empty pBabe vector (Mock/MEF) were analyzed by western blot. As expected, NC7 antibody, raised against a C-terminal peptide of full-length $\alpha 9$, only detected a 150kDa protein in $\alpha 9$ /MEF cell lysate. A 70kDa of SF $\alpha 9$ was detected in lysates of all SF $\alpha 9$ -expressing MEF cells by anti- $\alpha 9$ integrin (R&D) polyclonal antibody, raised against the extracellular domain of $\alpha 9$ integrin. A similar band was detected by anti-Myc antibody in lysates of SF $\alpha 9$ -Myc/MEF and by anti-FLAG antibody in lysates of SF $\alpha 9$ -FLAG/MEF. No immunoreactive bands were detected in culture supernatant of any SF $\alpha 9$ -expressing cell lines (Fig.3A), indicating that SF $\alpha 9$ is not secreted as a soluble protein. Anti-osteopontin antibody was used as a loading control in culture supernatant because MEF cells secrete osteopontin [22]. We then performed flow cytometry with anti- $\alpha 9$ integrin antibody Y9A2 and found surface expression of SF $\alpha 9$ in all SF $\alpha 9$ -expressing MEF cells (Fig.3B). To confirm surface expression of SF $\alpha 9$, we labeled cell surface proteins with a thiol-cleavable amine-reactive biotinylation reagent, sulfo-NHS-SS-Biotin. Biotin-labeled cells were lysed and captured with streptavidin agarose and labeled proteins were eluted by reduction and analyzed by immunoblotting. SF $\alpha 9$, like full-length $\alpha 9$ integrin was detectable in the avidin binding fraction (Fig.3C).

Having shown that SF $\alpha 9$ is expressed on the cell surface we assessed its ability to support cell adhesion to TNfn3RAA. SF $\alpha 9$ /MEF, SF $\alpha 9$ -Myc/MEF and SF $\alpha 9$ -FLAG/MEF cells did not bind to TNfn3RAA. $\alpha 9$ /MEF cells, expressing full-length $\alpha 9$, were used as positive control. Cell adhesion on the irrelevant substrate, plasma fibronectin, was similar among all

5 cell lines (Fig.3D). These findings indicated that SF α 9 by itself neither supports α 9-dependent cell adhesion, nor modulates RGD-dependent cell adhesion.

SF α 9 enhance adhesion mediated by full-length α 9 integrin

To test hypothesis that SF α 9 modulates α 9 β 1 integrin function, we established MEF cells stably expressing both full-length α 9 integrin and SF α 9 (SF α 9/ α 9/MEF, SF α 9-Myc/ α 9/MEF or SF α 9-FLAG/ α 9/MEF). Flow cytometry demonstrated similar surface levels of α 9 integrin (Fig.4A). Full-length α 9 integrin subunit and SF α 9 expression levels were estimated by western blot using anti- α 9 integrin antibody NC7, R&D, anti-Myc antibody or anti-FLAG antibody. We detected similar levels of α 9 integrin subunit in all MEF cells and SF α 9 in SF α 9/MEF, SF α 9-Myc/MEF, or SF α 9-FLAG/MEF cells (Fig.4B). These data suggest that co-expression of SF α 9 does not change full-length α 9 integrin expression. MEF cells expressing both SF α 9 and full-length α 9 integrin demonstrated enhanced cell adhesion compared to MEF cells only expressing full-length α 9 integrin (Fig.4C), suggesting that SF α 9 promotes α 9-dependent cell adhesion.

To further investigate the importance of endogenous SF α 9 for α 9-dependent cell adhesion, we examined endogenous SF α 9 expression in various tumor cell lines whose expression of full-length α 9 integrin or SF α 9 was tested by PCR and flow cytometry (Fig.2A–D). Endogenous SF α 9 protein was evaluated by first determining that of the 3 monoclonal antibodies we generated against human α 9 integrin A9A1 was most efficient for SF α 9 immunoprecipitation (Fig.5A). A9A1 was then used to immunoprecipitate lysates of SF α 9/ α 9/SW480 cells or various tumor cell lines. Two bands of 150kDa and 70kDa that correspond to full-length α 9 integrin and SF α 9, respectively, were detected by immunoblotting in SF α 9/ α 9/SW480 cells (Fig.5B). The band representing full-length α 9 integrin was detected in all cell lines but not A549 cell, whereas SF α 9 was detected in only RD cells. RD cells strongly bind to TNfn3RAA (Fig.2E) [17]. Therefore, we knocked down endogenous SF α 9 in RD cells with lentiviral vectors expressing small hairpin RNAs (shRNAs) based on the unique 3' sequence of SF α 9 derived from exon15b, which is not contained in full-length α 9 integrin mRNA, to investigate the importance of endogenous SF α 9 for α 9-dependent cell adhesion. Lentiviral transduction efficiency was monitored by GFP expression. Over 95% of RD cells transduced with each of two shRNAs were GFP⁺ (Fig.5C). Real-time PCR showed that SF α 9 shRNA 1 or 2 caused >80% or >40 reduction, respectively, in SF α 9 mRNA (Fig.5D) with no effect on full-length α 9 integrin mRNA expression. Western blotting confirmed that the expression of SF α 9 protein was substantially reduced in the cells transduced with SF α 9 shRNA1 with no effect on protein expression of full-length α 9 integrin (Fig.5E). RD cells transduced with shRNA1 manifested decreased cell adhesion on TNfn3RAA compared to RD cells transduced with empty pSicoR vector (Fig.5F), with noted no effect on adhesion to the irrelevant substrate, plasma fibronectin.

SF α 9 promotes full-length α 9 integrin-dependent cell adhesion via the authentic α 9 subunit cytoplasmic domain

To determine whether the authentic α 9 cytoplasmic domain is critical for SF α 9-dependent enhancement of cell adhesion, we transfected FLAG-tagged SF α 9 into CHO cells stably expressing wild type or chimeric α 9 subunits containing the α 9 extracellular and transmembrane domains and either authentic α 9 or α 4 or α 5 integrin cytoplasmic domains. SF α 9 expression did not change the expression level of α 9 integrin subunits in any of these CHO cell lines (Fig.6A). SF α 9-FLAG/ α 9/CHO cells demonstrated enhanced cell adhesion on TNfn3RAA compared to Mock/ α 9/CHO cells. However, SF α 9-FLAG/ α 9 α 5/CHO or SF α 9-FLAG/ α 9 α 4/CHO cells had no enhancement of cell adhesion (Fig.6B). As expected, expression of SF α 9 had no effect on cell adhesion to plasma fibronectin in any cell line (Fig.

6B). Thus, SF α 9 specifically promotes α 9 integrin-dependent cell adhesion through a mechanism that requires the α 9 subunit cytoplasmic domain.

SF α 9 binds to neither full-length α 9 subunit nor β 1 subunit

The full-length α 9 integrin subunit associates with the β 1 subunit. SF α 9 promotes full-length α 9-dependent cell adhesion. Therefore, we asked whether SF α 9 associates with α 9 or β 1 subunits to analyze the mechanism of cell surface expression and function of SF α 9. Lysates from Mock/ α 9/CHO cells or SF α 9-FLAG/ α 9/CHO cells were immunoprecipitated with anti-FLAG antibody or anti- α 9 antibody NC7, and immunoblotted with anti-FLAG antibody, anti- α 9 antibody NC7 or anti- β 1 integrin antibody. We found that neither the α 9 integrin subunit nor the β 1 subunit could be co-immunoprecipitated with anti-FLAG antibody (Fig. 7). Thus, SF α 9 does not bind full-length α 9 or β 1 integrin subunits.

Discussion

In the present study, we report the expression and function of a novel alternatively spliced variant of α 9 integrin, SF α 9. Whereas SF α 9 by itself does not mediate cell adhesion to the α 9 β 1 ligand, TNfn3RAA (Fig.3D), it does enhance adhesion in cells co-expressing full-length α 9 integrin (Fig.4C and 6B). Surprisingly, this effect requires the presence of specific sequences in the α 9 cytoplasmic domain (Fig.6B).

One potential explanation for the adhesion enhancing effects of SF α 9 would be that this variant associates with another transmembrane protein, which is not full-length α 9, or β 1 integrin subunits (Fig.7), and initiates signals that increase the avidity of the α 9 β 1 integrin for its ligands, a process that has been called inside-out signaling. There are numerous precedents for integrin activation by inside-out signal. For example, CXCR2, CXCR4, CCR3, formyl peptide receptor, IgE receptor, and IL-5 receptor modulate α 4 β 1 integrin binding [23, 24]. Platelet activators such as thrombin, and ATP modulate α IIb β 3 binding [25, 26]. Insulin-Like Growth Factor I modulates α v β 3 binding [27].

It has been reported recently that the src family kinase inhibitor PP1 inhibits α 9 integrin-dependent cell adhesion, suggesting inside-out activation of this integrin is regulated by src or other tyrosine kinases that can be inhibited by PP1 [21]. However, this effect is not α 9 integrin-specific because PP1 can inhibit cell adhesion to plasma fibronectin [28, 29].

Several integrin subunits have alternative splicing variants including α 3, α 6, α 7, α IIb, β 1, β 3, β 4, and β 5 [30, 31]. At least four integrin subunits, α 6, α 7, α IIb and β 3 have alternative splicing variant in their extracellular domains. In the α 6 and α 7 subunits alternative splicing inserts a new exon [32, 33], whereas in α IIb and β 3 there are truncated alternative splicing variants [34–36], similar to what we now describe for SF α 9. Both truncated α IIb and β 3 lack transmembrane and cytoplasmic domains, and have novel C-terminal amino acid sequences. However, unlike SF α 9, both are secreted protein. Secreted truncated β 3 inhibits cell adhesion [35]. Thus SF α 9 has the unique features of cell surface expression and of promotion of cell adhesion.

Some integrins α subunits (α 3, α 4, α 5, α 6, α 7, α 8, α v, α E, and α IIb) undergo post-translational endoproteolytic cleavage within their extracellular domains [37]. α 4, which is a structurally related to α 9, is cleaved into N-terminal (80kDa) and C-terminal (70kDa) fragments at Arg 597 by furin [37]. Although the N-terminal cleaved product has no transmembrane domain, this product is expressed on the cell surface [37]. Both SF α 9 and the N-terminal cleaved product of α 4 are principally composed of the β -propeller and partially thigh domains, and are expressed on cell surface. In α 6 and α 7, the N-terminal cleaved product has β -propeller and partial thigh domains. Processing of α 6 and α 7 is

important for inside-out signaling and enhanced cell adhesion, respectively [38, 39], similar to the results described here.

We evaluated full-length $\alpha 9$ integrin expression by PCR (Fig.2A, 2B), flow cytometry (Fig. 2D), and western blotting (Fig.5B). LN-229, G361, RD, and MDA-MB-435s express full-length $\alpha 9$ integrin, but only RD cells can bind to TNfn3RAA strongly (Fig.2E). We also evaluated SF $\alpha 9$ expression in several cell lines by PCR (Fig.2A, 2B) and western blotting (Fig.5B). SF $\alpha 9$ mRNA was found in all cells expressing full-length $\alpha 9$ integrin protein, but SF $\alpha 9$ protein was found in only RD cells. These findings stimulated us to knockdown SF $\alpha 9$ in RD cells. Knockdown of SF $\alpha 9$ in RD cells decreased $\alpha 9\beta 1$ -mediated cell adhesion (Fig. 5F). These results suggest that SF $\alpha 9$ modulates the function of full-length $\alpha 9$ integrin.

To analyze the mechanism by which SF $\alpha 9$ is expressed on the cell surface and modulates the function of full-length $\alpha 9$ integrin, we examined whether SF $\alpha 9$ associates with full-length $\alpha 9$ integrin or $\beta 1$ integrin subunits. We found that SF $\alpha 9$ does not associate with $\alpha 9$ or $\beta 1$. Further experiments are needed to determine how SF $\alpha 9$ is expressed on the cell surface and how it promotes $\alpha 9$ -dependent cell adhesion.

In summary, SF $\alpha 9$, which is a truncated isoform of the $\alpha 9$ integrin subunit containing only the β -propeller domain, thigh domain and an additional 19 amino acids derived from an intron, is expressed on the cell surface, but cannot bind $\alpha 9$ integrin ligand, TNfn3RAA. Over-expression of SF $\alpha 9$ in cells expressing full-length $\alpha 9$ promotes $\alpha 9$ -dependent cell adhesion mediated through the cytoplasmic domain of the co-expressed full-length $\alpha 9$ subunit. Thus, our findings suggest that SF $\alpha 9$ is a novel functional modulator of the $\alpha 9\beta 1$ integrin.

The abbreviations used are

SF$\alpha 9$	short form of $\alpha 9$ integrin
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
ADAM	a disintegrin and metalloprotease
SSAT	spermidine/spermin N^1 -acetyltransferase
Kir	inward-rectifying K ⁺ (channel)
CHO	chinese hamster ovary
MEF	mouse embryonic fibroblast
TNfn3RAA	the mutant form of the third fibronectin type III repeat in tenascin-C
BSA	bovine serum albumin
DTT	dithiothreitol
shRNA	small hairpin RNA

Acknowledgments

We thank Michael McManus (University of California, San Francisco) for shRNA construction and lentiviral infection assistance. We also thank Chun Chen and Ahnika Kline for helpful suggestions and technical help. This work was supported by National Institutes of Health Grant HL64353 (to D.S.), and the Uehara memorial foundation (to SK).

References

1. Hynes RO. Integrins: a family of cell surface receptors. *Cell*. 1987; 48:549–554. [PubMed: 3028640]
2. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*. 1992; 69:11–25. [PubMed: 1555235]
3. Springer TA. Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. *Proc. Natl. Acad. Sci. U. S. A.* 1997; 94:65–72. [PubMed: 8990162]
4. Diamond MS, Springer TA. The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 1994; 4:506–517. [PubMed: 7922371]
5. Ginsberg MH. Integrins: dynamic regulation of ligand binding. *Biochem. Soc. Trans.* 1995; 23:439–446. [PubMed: 8566327]
6. Vlahakis NE, Young BA, Atakilit A, Hawkridge AE, Issaka RB, Boudreau N, Sheppard D. Integrin alpha9beta1 directly binds to vascular endothelial growth factor (VEGF)-A and contributes to VEGF-A-induced angiogenesis. *J. Biol. Chem.* 2007; 282:15187–15196. [PubMed: 17363377]
7. Vlahakis NE, Young BA, Atakilit A, Sheppard D. The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. *J. Biol. Chem.* 2005; 280:4544–4552. [PubMed: 15590642]
8. Taooka Y, Chen J, Yednock T, Sheppard D. The integrin alpha9beta1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. *J. Cell Biol.* 1999; 145:413–420. [PubMed: 10209034]
9. Yokosaki Y, Matsuura N, Sasaki T, Murakami I, Schneider H, Higashiyama S, Saitoh Y, Yamakido M, Taooka Y, Sheppard D. The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J. Biol. Chem.* 1999; 274:36328–36334. [PubMed: 10593924]
10. Eto K, Huet C, Tarui T, Kupriyanov S, Liu HZ, Puzon-McLaughlin W, Zhang XP, Sheppard D, Engvall E, Takada Y. Functional classification of ADAMs based on a conserved motif for binding to integrin alpha 9beta 1: implications for sperm-egg binding and other cell interactions. *J. Biol. Chem.* 2002; 277:17804–17810. [PubMed: 11882657]
11. Chen C, Young BA, Coleman CS, Pegg AE, Sheppard D. Spermidine/spermine N1-acetyltransferase specifically binds to the integrin alpha9 subunit cytoplasmic domain and enhances cell migration. *J. Cell Biol.* 2004; 167:161–170. [PubMed: 15479742]
12. deHart GW, Jin T, McCloskey DE, Pegg AE, Sheppard D. The alpha9beta1 integrin enhances cell migration by polyamine-mediated modulation of an inward-rectifier potassium channel. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:7188–7193. [PubMed: 18480266]
13. Young BA, Taooka Y, Liu S, Askins KJ, Yokosaki Y, Thomas SM, Sheppard D. The cytoplasmic domain of the integrin alpha9 subunit requires the adaptor protein paxillin to inhibit cell spreading but promotes cell migration in a paxillin-independent manner. *Mol. Biol. Cell.* 2001; 12:3214–3225. [PubMed: 11598204]
14. Liu S, Slepak M, Ginsberg MH. Binding of Paxillin to the alpha 9 Integrin Cytoplasmic Domain Inhibits Cell Spreading. *J. Biol. Chem.* 2001; 276:37086–37092. [PubMed: 11477105]
15. Swift S, Lorens J, Achacoso P, Nolan GP. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. Chapter 10. *Curr. Protoc. Immunol.* 2001 Unit 10 17C.
16. Wang A, Yokosaki Y, Ferrando R, Balmes J, Sheppard D. Differential regulation of airway epithelial integrins by growth factors. *Am. J. Respir. Cell. Mol. Biol.* 1996; 15:664–672. [PubMed: 8918373]
17. Yokosaki Y, Palmer EL, Prieto AL, Crossin KL, Bourdon MA, Pytela R, Sheppard D. The integrin alpha 9 beta 1 mediates cell attachment to a non-RGD site in the third fibronectin type III repeat of tenascin. *J. Biol. Chem.* 1994; 269:26691–26696. [PubMed: 7523411]
18. Yokosaki Y, Matsuura N, Higashiyama S, Murakami I, Obara M, Yamakido M, Shigeto N, Chen J, Sheppard D. Identification of the ligand binding site for the integrin alpha9 beta1 in the third fibronectin type III repeat of tenascin-C. *J. Biol. Chem.* 1998; 273:11423–11428. [PubMed: 9565552]

19. Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R, Jacks T. Cre-lox-regulated conditional RNA interference from transgenes. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:10380–10385. [PubMed: 15240889]
20. Brown MC, Staniszevska I, Lazarovici P, P.Tuszynski G, Del Valle L, Marcinkiewicz C. Regulatory effect of nerve growth factor in alpha9beta1 integrin-dependent progression of glioblastoma. *Neuro. Oncol.* 2008; 10:968–980. [PubMed: 19074980]
21. Lydolph MC, Morgan-Fisher M, Hoye AM, Couchman JR, Wewer UM, Yoneda A. alpha9beta1 Integrin in melanoma cells can signal different adhesion states for migration and anchorage. *Exp. Cell. Res.* 2009; 315:3312–3324. [PubMed: 19796635]
22. Wu Y, Denhardt DT, Rittling SR. Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. *Br. J. Cancer.* 2000; 83:156–163. [PubMed: 10901364]
23. Chigaev A, Blenc AM, Braaten JV, Kumaraswamy N, Kepley CL, Andrews RP, Oliver JM, Edwards BS, Prossnitz ER, Larson RS, Sklar LA. Real time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. *J. Biol. Chem.* 2001; 276:48670–48678. [PubMed: 11641394]
24. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2002; 283:R7–R28. [PubMed: 12069927]
25. Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 1979; 254:5357–5363. [PubMed: 447655]
26. Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J. Biol. Chem.* 1980; 255:154–161. [PubMed: 7350149]
27. Sekimoto H, Eipper-Mains J, Pond-Tor S, Boney CM. (alpha)v(beta)3 integrins and Pyk2 mediate insulin-like growth factor I activation of Src and mitogen-activated protein kinase in 3T3-L1 cells. *Mol. Endocrinol.* 2005; 19:1859–1867. [PubMed: 15761030]
28. Butler B, Blystone SD. Tyrosine phosphorylation of beta3 integrin provides a binding site for Pyk2. *J. Biol. Chem.* 2005; 280:14556–14562. [PubMed: 15695828]
29. Wang Y, Jin G, Miao H, Li JY, Usami S, Chien S. Integrins regulate VE-cadherin and catenins: dependence of this regulation on Src, but not on Ras. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:1774–1779. [PubMed: 16446427]
30. Fornaro M, Languino LR. Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol.* 1997; 16:185–193. [PubMed: 9402008]
31. de Melker AA, Sonnenberg A. Integrins: alternative splicing as a mechanism to regulate ligand binding and integrin signaling events. *Bioessays.* 1999; 21:499–509. [PubMed: 10402956]
32. Delwel GO, Kuikman I, Sonnenberg A. An alternatively spliced exon in the extracellular domain of the human alpha 6 integrin subunit--functional analysis of the alpha 6 integrin variants. *Cell Adhes. Commun.* 1995; 3:143–161. [PubMed: 7583007]
33. Ziober BL, Chen Y, Kramer RH. The laminin-binding activity of the alpha 7 integrin receptor is defined by developmentally regulated splicing in the extracellular domain. *Mol. Biol. Cell.* 1997; 8:1723–1734. [PubMed: 9307969]
34. Trikha M, Cai Y, Grignon D, Honn KV. Identification of a novel truncated alphaIIb integrin. *Cancer Res.* 1998; 58:4771–4775. [PubMed: 9809974]
35. Jin R, Trikha M, Cai Y, Grignon D, Honn KV. A naturally occurring truncated beta3 integrin in tumor cells: native anti-integrin involved in tumor cell motility. *Cancer Biol. Ther.* 2007; 6:1559–1568. [PubMed: 17912026]
36. Djaffar I, Chen YP, Creminon C, Maclouf J, Cieutat AM, Gayet O, Rosa JP. A new alternative transcript encodes a 60 kDa truncated form of integrin beta 3. *Biochem. J.* 1994; 300(Pt 1):69–74. [PubMed: 8198553]
37. Bergeron E, Basak A, Decroly E, Seidah NG. Processing of alpha4 integrin by the proprotein convertases: histidine at position P6 regulates cleavage. *Biochem. J.* 2003; 373:475–484. [PubMed: 12691605]
38. Liu J, Guppur PB, Kaufman SJ. Genetically determined proteolytic cleavage modulates alpha7beta1 integrin function. *J. Biol. Chem.* 2008; 283:35668–35678. [PubMed: 18940796]

39. Delwel GO, Kuikman I, van der Schors RC, de Melker AA, Sonnenberg A. Identification of the cleavage sites in the alpha6A integrin subunit: structural requirements for cleavage and functional analysis of the uncleaved alpha6Abeta1 integrin. *Biochem. J.* 1997; 324(Pt 1):263–272. [PubMed: 9164866]

Fig. 1. Short form of $\alpha 9$ structure. (A) Schematic representation of $\alpha 9$ integrin and SF $\alpha 9$ protein. The β -propeller, thigh, genu, calf-1, calf-2, transmembrane (TM), and cytoplasmic (CYT) domains are indicated. The SF $\alpha 9$ protein is identical to $\alpha 9$ integrin up to thigh domain and contains 19 novel amino acids. (B) Schematic representation of $\alpha 9$ integrin and SF $\alpha 9$ genome and cDNA. The cDNA sequence coded 19 novel amino acids in SF $\alpha 9$ are derived from exon 15b. (C) The cDNA sequence of SF $\alpha 9$ derived from exon 15b. The border between exon 15a and 15b has a GT splice donor site. 3' end has poly(A)⁺ tail. The polyadenylation cleavage site (AATAAA) is indicated (arrowhead).

Fig.2.

Expression of full-length $\alpha 9$ integrin subunit or SF $\alpha 9$ in various tumor cells. RT-PCR analysis (A) or real-time PCR analysis (B) of full-length $\alpha 9$ integrin or SF $\alpha 9$ expression in various tumor cells. (C) Absolute copy number of full-length $\alpha 9$ or SF $\alpha 9$ mRNA. (D) Surface expression of $\alpha 9$ on various tumor cells. Flow cytometry demonstrating cell surface expression of $\alpha 9$ integrin subunit. Shaded peaks represent unstained cells, and open peaks represent cells stained with anti- $\alpha 9$ integrin Y9A2. (E) Cell binding of various tumor cells to TNfn3RAA (20 μ g/ml). Data are presented as means $\pm$ SEM of 3 independent experiments.

Fig. 3.

Expression and adhesion of SFα9. (A) SFα9 is not secreted as a soluble protein. Western blot analysis of full-length α9 integrin subunit and SFα9 in cell lysates and culture supernatant from MEF cells transduced with retroviruses encoding the cDNAs of α9 integrin subunit, SFα9, Myc-tagged, FLAG-tagged SFα9, or control. Cell culture supernatants were concentrated 10-fold using centricon tubes (Millipore). Anti-β-actin antibody or anti-osteopontin antibody was used as loading control for cell lysate or culture supernatant, respectively. (B, C) SFα9 is expressed on cell surface. Flow cytometry demonstrating cell surface expression of α9 integrin subunit or SFα9. Shaded peaks represent unstained cells, and open peaks represent cells stained with anti-α9 integrin Y9A2 (B). Cell surface proteins were labeled with thiol-cleavable biotin, captured with streptavidin agarose, then eluted by reduction. Eluted proteins were then analyzed by western blotting (C). (D) SFα9 does not bind to TNfn3RAA (tenascin-C). The binding of MEF cells expressing α9 integrin subunit or SFα9 to the indicated concentrations of TNfn3RAA and fibronectin. Asterisk represents a statistical significant difference (*P < 0.05; **P < 0.005) as compared with adhesion of Mock/MEF cells. Data are presented as means ± SEM of 3 independent experiments.

Fig. 4.

SFα9 promotes α9-dependent cell adhesion. (A) Flow cytometric evaluation of cell surface expression of α9 integrin subunit on MEF cells co-transduced with α9 integrin subunit and two different-tagged or non-tagged SFα9. (B) Western blot analysis of full-length α9 integrin subunit and SFα9 in lysates from MEF cells co-transduced with full-length α9 integrin subunit and two different-tagged or non-tagged SFα9. (C) The binding of MEF cells co-expressing full-length α9 integrin subunit and two different-tagged or non-tagged SFα9 to the indicated concentration of TNfn3RAA and fibronectin. Asterisk represents a statistical significant difference (*P < 0.05; **P < 0.005) as compared with adhesion of Mock/α9/MEF cells. Data are presented as means ± SEM of 4 independent experiments.

Fig. 5.

Endogenously expressed SF α 9 promotes α 9-dependent cell adhesion. (A) A9A1 is useful antibody for SF α 9 immunoprecipitation. Lysates from SF α 9-FLAG/ α 9/SW480 cells were immunoprecipitated by anti- α 9 integrin Y9A2, A9A1, or B9A1, precipitated proteins were then separated by SDS-PAGE, and blotted with anti-FLAG antibody. Anti-FLAG antibody for immunoprecipitation was used as positive control. (B) Endogenous expression of SF α 9 in RD cells. Lysates from SF α 9-FLAG/ α 9/SW480 cells or various tumor cell lines were immunoprecipitated by anti- α 9 integrin A9A1, precipitated proteins were separated by SDS-PAGE, and blotted with anti- α 9 antibody (R&D). (C) Flow cytometric evaluation of GFP expression in RD cells transduced with lentiviruses encoding the SF α 9 shRNA at 4 days after transduction. (D) Full-length α 9 integrin subunit and SF α 9 mRNA levels in SF α 9 shRNA-transduced RD cells at 4 days after transduction was quantified using real-time PCR and expressed as percent basal levels (normalized to RD cells transduced with pSicoR vector). Asterisk represents a statistical significant difference (* $P < 0.05$; ** $P < 0.005$) as compared with expression of SF α 9 in RD cells transduced with pSicoR vector. (E) Full-length α 9 integrin subunit and SF α 9 protein in SF α 9 shRNA-transduced RD cells at 4 days after transduction was detected by immunoprecipitation, as described in (B). (F) Cell adhesion of SF α 9 shRNA-transduced RD cells at 4 days after transduction to TNfn3RAA or plasma fibronectin. Asterisk represents a statistical significant difference (* $P < 0.05$; ** $P < 0.005$) as compared with adhesion of RD cells transduced with pSicoR vector. Data are presented as means $\pm$ SEM of 3 independent experiments.

Fig. 6.

Effect of $\alpha 9$ integrin subunit cytoplasmic domain on promoting $\alpha 9$ -dependent cell adhesion by SF $\alpha 9$. (A) Flow cytometry of cell surface expression of $\alpha 9$ integrin subunit on $\alpha 9$ -, $\alpha 9\alpha 5$ -, $\alpha 9\alpha 4$ -expressing CHO cells transfected with FLAG-tagged SF $\alpha 9$. Shaded peaks represent unstained cells, and open peaks represent cells stained with anti- $\alpha 9$ integrin Y9A2. (B) Adhesion of $\alpha 9$ -, $\alpha 9\alpha 5$ -, $\alpha 9\alpha 4$ -expressing CHO cells transfected with FLAG-tagged SF $\alpha 9$ to TNfn3RAA or plasma fibronectin. Asterisk represents a statistical significant difference (* $P < 0.05$; ** $P < 0.005$) as compared with adhesion of Mock/ $\alpha 9$ /CHO, Mock/ $\alpha 9\alpha 5$ /CHO, or Mock/ $\alpha 9\alpha 4$ /CHO cells. Data are presented as means $\pm$ SEM of 3 independent experiments.

Fig. 7.

SFα9 binds to neither full-length α9 integrin subunit nor β1 integrin subunit. Lysates from CHO cells co-expressing full-length α9 integrin subunit and FLAG-tagged SFα9 or control were immunoprecipitated by anti-FLAG antibody or anti-α9 integrin (NC7) antibody. The precipitated proteins were then separated by SDS-PAGE, and blotted with anti-FLAG, NC7, or anti-β1 integrin antibodies. The whole cell lysates (WCL) were immunoblotted with anti-FLAG, NC7, or anti-β1 integrin antibodies.