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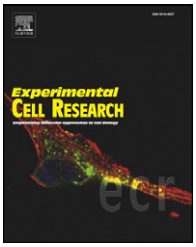

Research Article

Short form of α9 promotes α9β1 integrin-dependent cell adhesion by modulating the function of the full-length α9 subunit

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ABSTRACT

The α9β1 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 α9 integrin subunit cDNA was identified by searching the Medline database. This splice variant, which we called the short form of α9 integrin (SFα9), encodes a 632-aa isoform lacking transmembrane and cytoplasmic domains, and its authentic expression was verified by PCR and Western blotting. SFα9 is expressed on the cell surface but cannot bind ligand in the absence of the full-length α9 subunit. Over-expression of SFα9 in cells expressing full-length α9 promotes α9-dependent cell adhesion. This promoting effect of SFα9 requires the authentic cytoplasmic domain of the co-expressed full-length α9 subunit. Thus, SFα9 is a novel functional modulator of α9β1 integrin by inside-out signaling.

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 αβ 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 as 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].

The α9β1 integrin is widely expressed and binds to a variety of ligands, including tenascin-C; osteopontin; VCAM-1; VEGF-A, -C, and -D; and several members of the ADAM family [6–10]. At least two proteins that directly interact with the α9 integrin subunit cytoplasmic domain have been reported to modulate α9β1-

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Abbreviations: SFα9, short form of α9 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

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mediated cell migration and cell spreading. SSAT (spermidine/spermin N^1-acetyltransferase) binds to the integrin α9 cytoplasmic domain and modulates inward rectification of the Kir4.2 (inward-rectifier K+) channel to enhance α9 integrin-dependent cell migration [11,12]. Paxillin binding to the integrin α9 cytoplasmic domain mediates inhibition of cell spreading by α9β1 [13,14]. However, no molecule has yet been identified as a specific modifier of integrin α9-dependent adhesion.

We found a sequence predicted to encode an alternative splice variant of the α9 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 α9 (SFα9) 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 α9β1 ligands, SFα9 enhances adhesion mediated by full-length α9 integrin, an effect that depends on the presence of sequences in the cytoplasmic domain of the full-length α9 integrin.

Materials and methods

Cell culture and reagents

CHO cells, MEF cells, SW480 cells, human Phoenix-E (φE) 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), and MDA-MB-435 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-α9 integrin antibodies Y9A2 [16], A9A1 [7], or B9A1 for flow cytometry or immunoprecipitation were generated and characterized in our laboratory. Anti-α9 integrin monoclonal antibody NC7 for Western blotting was generated by immunizing mice with a peptide derived from the C-terminal domain of mouse α9, EAEKNRKENEDGWDWVQKNQ. Anti-FLAG antibody M2 and anti-Myc antibody 9E10 were obtained from Sigma-Aldrich and Roche Applied Science, respectively. Anti-β1 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.

SFα9 cDNA cloning

The cDNAs for the alternative splicing variant of α9 integrin (SFα9), FLAG-tagged SFα9, and Myc-tagged SFα9 cDNA were PCR amplified from G361 human melanoma cells using the following primers: 5′-CGGTACACCTACCTGGGCTA-3′ (sense) and 5′-AGGAGTCGACAAAGGGAGACCCAGAGAGGA-3′ (antisense for SFα9), 5′-CCGTCGTCGACCTATTTATCGTCATCATCTTTGTAGTCTTTCTGCTCTGTGCAGCTGCC-3′ (antisense for FLAG-tagged SFα9), and 5′-CCGTCGTCGACTTACAGGTCTTCCTCAGAGATGAGTTTCTGCTCTGTGCAGCTGCC-3′ (antisense for Myc-tagged SFα9). PCR products were digested with BglII and SalI then inserted into BglII and SalI-digested α9 integrin expressing vector, α9-pBabepuro. A fragment digested with BglII and SalI from Myc-tagged SFα9-pBabepuro was inserted into BglII and SalI-digested α9 integrin expressing vector, α9-pWZLblast.

Generation of stable cell lines

MEF cells expressing α9 integrin subunit or SFα9 were generated as follows. α9-pBabepuro or SFα9-pBabepuro were transfected into φE packaging cells by Lipofectamine 2000 (Invitrogen). Three days after transfection, virus-containing supernatants were harvested and filtered through a 0.45-μm filter and then added to 50% confluent MEF cells in the presence of 8 μg/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 μg/ml puromycin (Sigma-Aldrich). For generation of MEF cells co-expressing α9 integrin subunit and SFα9, retroviruses were generated by transfecting α9-pWZLblast then added to SFα9/MEF, SFα9-Myc/MEF, or SFα9-FLAG/MEF cells. MEF cells transduced by this virus were cultured in 10% FCS DMEM supplemented with 10 μg/ml puromycin and 10 μg/ml Blasticidin (Invitrogen). MEF cells expressing the α9 integrin subunit and/or SFα9 were identified by flow cytometry with the anti-α9 antibody Y9A2, and Western blotting, with anti-α9 antibody (NC7 or AF3827 from R&D Systems), anti-Myc antibody, and anti-FLAG antibody. α9-, α9α5, α9α4 integrin-expressing CHO cells, and α9-expressing SW480 cells were previously generated in our laboratory [13, 17]. FLAG-tagged SFα9 was transfected into CHO cells or α9-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 h at room temperature. Cells were suspended in DMEM containing 0.25% BSA and 200 μl of cell suspension (at a cell density of 5×10^4 cells per well) was applied to 96-well plates and incubated for 1 h 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 3 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 α9 integrin, and SFα9 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× protease inhibitors (1× 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-α9 antibody NC7, anti-FLAG antibody (for CHO cells), or anti-α9 antibody Y9A2, A9A1, or B9A1 (for SW480 or various tumor cell lines) at 4 °C, 1 h. The beads were washed with the same buffer five times, and precipitated