Spermidine/Spermine $N^1$-Acetyltransferase specifically binds to the integrin $\alpha_9$ subunit cytoplasmic domain and enhances cell migration

Chun Chen$^1$, Bradford A. Young$^1$, Catherine S. Coleman$^2$, Anthony E. Pegg$^2$ and Dean Sheppard$^1$

1. Lung Biology Center, Department of Medicine, University of California, San Francisco, San Francisco, California 94110
2. Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Correspondence should be addressed to D. S. (E-mail: deans@itsa.ucsf.edu).

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Abstract

The integrin α9β1 is expressed on migrating cells, such as leukocytes, and binds to multiple ligands present at sites of tissue injury and inflammation. α9β1, like the structurally-related integrin, α4β1, mediates accelerated cell migration, an effect that depends on the α9 cytoplasmic domain. α4β1 enhances migration through reversible binding to the adaptor protein, paxillin, but α9β1-dependent migration is paxillin independent. Using yeast two-hybrid screening, we identified the polyamine catabolizing enzyme, spermidine/spermine N1-acetyltransferase (SSAT) as a specific binding partner of the α9 cytoplasmic domain. Over-expression of SSAT increased α9β1-mediated migration, and siRNA knockdown of SSAT inhibited this migration without affecting cell adhesion or migration mediated by other integrin cytoplasmic domains. The enzyme activity of SSAT is critical for this effect, since a catalytically inactive version did not enhance migration. We conclude that SSAT directly binds to the α9 cytoplasmic domain and mediates α9-dependent enhancement of cell migration, presumably by localized effects on acetylation of polyamines or as yet unidentified substrates.
Cell migration is central to many biological and pathological processes, including embryogenesis, inflammation, tissue repair and regeneration, and cancer metastasis (Lauffenburger and Horwitz, 1996). For cells to migrate on a substrate, they must adhere and de-adhere in a coordinated manner and generate tensile force in the direction of migration. The force required to promote migration is generated by the actin cytoskeleton and integrin-dependent protein complexes that anchor actin to specific sites on the cell membrane (Lauffenburger and Horwitz, 1996).

While several members of the integrin family can mediate cell migration, two structurally-related integrin α subunits, α4 and α9 appear to play specialized roles in accelerated migration. Several studies utilizing chimeric integrin α subunits have demonstrated that this enhancement of migration depends on distinct sequences within the cytoplasmic domains of α4 (Chan et al., 1992; Kassner et al., 1995; Kassner and Hemler, 1993) and α9 (Young et al., 2001). Integrin α4-mediated cell migration depends on the specific interaction of the α4 cytoplasmic domain with the adapter protein paxillin (Liu et al., 1999), and requires the dissociation of paxillin from integrin at the leading edge of migrating cells upon phosphorylation of a specific serine (S) at position 988 in the α4 cytoplasmic domain (Goldfinger et al., 2003; Han et al., 2001). The α9 cytoplasmic domain is 47% identical to the α4 cytoplasmic domain, and also binds paxillin (Young et al., 2001). However, α9 does not contain a homologous phosphorylation site, and while the interaction between paxillin and α9 is critical for the effects of the integrin α9β1 on cell spreading, this interaction is completely dispensable for α9-dependent enhancement of cell migration (Young et al., 2001).
We therefore utilized yeast two-hybrid screening to identify proteins which interact with the α9 cytoplasmic domain to mediate enhanced cell migration. Using the entire cytoplasmic domain of human α9 (residues 974-1006) as bait, a human leukocyte cDNA library was screened. Several candidate cDNAs were isolated and sequenced. These candidates were re-screened by co-transformation with bait vectors containing the α9, α5, α4, α2 or β1 cytoplasmic domains. Only a single candidate: human spermidine/spermine N\(^1\)-acetyltransferase (SSAT) was found to specifically interact with α9 and not with α5, α4, α2 or β1 (Fig. 1a).

To confirm the results of the two-hybrid screen and to determine precisely which region(s) of SSAT interacted with the α9 cytoplasmic domain, we evaluated the binding of GST fusion proteins containing the α9, α5, α4 and α2 cytoplasmic domains (GST-α9, GST-α5, GST-α4 and GST-α2) to [35S] Met-labeled SSAT generated by in vitro transcription and translation. SSAT specifically bound to GST-α9 but not to α2, α4, α5 or GST alone (Fig. 1b). To map the binding site in SSAT, we generated a series of N terminal and C terminal truncation mutants and found that the C terminal (142aa-171aa) 30 residues including the catalytic site (Coleman et al., 1996) were not required for binding, but the 20 amino acids at the N terminus were critical (Fig. 1c). A double mutant R101A/E152K, that was previously shown to be catalytically inactive (Coleman et al., 1996) could also bind the α9 cytoplasmic domain (Fig. 1c).

To determine whether SSAT associates with α9 in cells, CHO cells stably expressing
either wild type α9 or chimeric α subunits containing the α9 extracellular and transmembrane domains fused to the cytoplasmic domains of either α4 or α5 (Young et al., 2001) were transfected to stably express SSAT-Myc. Flow cytometry demonstrated similar levels of α9, α9α4 and α9α5 expressed on the cell surface (Fig.2a). Lysates from these cell lines were immunoprecipitated with the anti-α9β1 antibody, Y9A2 (Wang et al., 1996) and immunoblotted with an anti-Myc antibody. Because endogenous and heterologously expressed SSAT have previously been shown to be rapidly degraded in CHO cells (McCloskey et al., 1999), the concentration of Myc-tagged SSAT was increased by treatment with the spermine analogue, \(N^{11}\),\(N^{11}\)-bis(ethyl)norspermine tetrahydrochloride (BE-3-3-3), which prevents proteosome-mediated SSAT degradation, as has been previously described (Coleman and Pegg, 2001). Western blotting of immunoprecipitates with anti-serum against the cytoplasmic domain of the integrin β1 subunit confirmed that similar amounts of α9β1 were precipitated from each lysate. SSAT was co-immunoprecipitated with full-length α9β1 in cells treated with BE-3-3-3, but no association with the α9α5 or α9α4 chimeras could be detected (Fig. 2b).

To determine if there is physical interaction between SSAT and α9 at the cell surface, cell surface proteins were labeled by a thiol-cleavable amine-reactive biotinylation reagent, Sulfo-NHS-SS-Biotin. Biotin-labeled cells were lysed and labeled proteins were captured by streptavidin agarose. Cell surface proteins were eluted from agarose by reduction and used for co-immunoprecipitation. SSAT was co-immunoprecipitated with full-length cell surface α9β1 from cells treated with BE-3-3-3, but no association with the α9α5 or α9α4 chimeras could be detected (Fig. 2c). Thus, SSAT associates with
α9β1 at the cell surface.

To determine if the interaction of SSAT and the α9 cytoplasmic domain is critical for α9-dependent enhancement of cell migration, we used Chinese hamster ovary (CHO) cells stably expressing wild type or chimeric α9 subunits (Young et al., 2001) for cell adhesion and migration assays on the α9β1-specific substrate, TNfn3RAA, in the presence of a range of concentrations of BE-3-3-3 to increase endogenous levels of SSAT, and in the presence or absence of the α9β1-blocking antibody, Y9A2. The expression of SSAT was induced by BE-3-3-3 in a dose-dependent manner (Fig. 3a). In the absence of BE-3-3-3, both the α9 and the α4 cytoplasmic domains caused similar enhancement of cell migration compared with the cytoplasmic domain of α5. However, BE-3-3-3 caused concentration-dependent enhancement of migration only in cells expressing wild type α9 (Fig. 3b). As we have previously reported (Young et al., 2001), all three versions of the α9 subunit supported equivalent adhesion to TNfn3RAA, and adhesion was unaffected by BE-3-3-3 treatment in all three cell lines (Fig. 3c). As expected adhesion and migration of all α9-expressing cells was inhibited by the anti-α9β1 antibody, Y9A2. Migration on the irrelevant substrate, plasma fibronectin, was similar for all 3 cell lines and was unaffected by treatment with BE-3-3-3 (Fig. 3d). Adhesion to fibronectin was also similar for all 3 cell lines and was unaffected by BE-3-3-3 (data not shown). These results indicate that the effects of BE-3-3-3 are specific for α9β1-mediated cell migration and are dependent on the presence of the α9 cytoplasmic domain.

Confluent CHO cells expressing wild-type α9 or α9α5 were plated on TNfn3RAA and
scratch wounded. Closure of the wound was quantified by phase microscopy as a measure of directed cell migration. Cells expressing the α9α5 chimera showed markedly slower migration and wound closure than cells expressing wild-type α9. Wound closure was accelerated by BE-3-3-3 in cells expressing wild-type α9, but not in cells expressing the α9α5 chimera (Fig. 4a). Photomicrographs of the wound edge did not reveal any systematic differences in cell morphology between cells expressing α9 in the presence or absence of treatment with BE-3-3-3 (Fig. 4b).

Endogenous levels of SSAT are below the level of detection by existing anti-SSAT antibodies. To confirm the relationship between the SSAT expression level and α9β1-dependent cell migration, and to determine whether the effects of SSAT depend on catalytic activity, we also performed adhesion and migration assays in CHO cells stably transfected to express either wild type or catalytically inactive (R101A/E152K mutant) c-Myc tagged SSAT. In the absence of BE-3-3-3, cells co-expressing wild type α9 and exogenous wild type SSAT demonstrated enhancement of cell migration on TNFα3RAA compared to cells expressing wild type α9 alone (Figure 5a), and cell migration was further enhanced by treatment with BE-3-3-3. In contrast, neither treatment with BE-3-3-3 nor over-expression of wild type SSAT had any effect on migration in cells expressing the chimeric subunits composed of the extracellular and transmembrane domains of α9 fused to the cytoplasmic domains of α4 (α9α4) or α5 (α9α5). The relative SSAT protein concentrations in mock- and SSAT-transfected cells in the presence and absence of treatment with BE 3-3-3 treated cells are shown in Figure 5d. Expression of catalytically inactive SSAT (Coleman et al., 1996) did not affect the
baseline enhancement of cell migration in cells expressing wild type α9, perhaps because
the level of expression was not adequate to effectively compete with endogenous SSAT.
However, this construct did not enhance cell migration and prevented even the increase in
migration caused by BE-3-3-3 in cells expressing wild type α9 alone, consistent with
previous reports that this construct can function as a dominant negative inhibitor of wild
type SSAT (Coleman et al., 1996). Similar to the previous experiments, over-expression
of either form of SSAT had no effects on cell adhesion on TNfn3RAA or on migration on
plasma fibronectin in the presence or absence of BE-3-3-3 (Fig. 5b and 5c). These
results confirm the specificity of SSAT for enhancement of α9-dependent cell migration
and suggest that this effect depends on the catalytic activity of SSAT.

To further investigate the importance of SSAT for α9β1-dependent enhancement of cell
migration, we used RNA interference to suppress the expression of SSAT in α9
expressing mouse embryonic fibroblasts (MEFs). MEFs were chosen because the
availability of complete genomic sequence data in the mouse allows improved design of
specific siRNA and mouse and human SSAT are virtually identical. Four 21-nucleotide
siRNA segments directed against the middle (siRNA-1 and siRNA-3) or the 3’ end
(siRNA-2 and siRNA-4) of the SSAT transcript were transfected into MEFs that had
been stably transfected to express either wild type α9, α9α4 or α9α5 and the cells were
examined 24 h later (Young et al., 2001). Real-time PCR showed that SSAT mRNA was
reduced by more than 75% by siRNA-2, siRNA-3 and siRNA-4 compared to
untransfected cells or cells transfected with siRNA-1 (Fig. 6a). Since endogenous SSAT
cannot be detected by polyclonal antibody against SSAT, BE-3-3-3 was used to induce
the expression of SSAT 24 h after siRNA transfection. The SSAT protein level was decreased by siRNA-2, siRNA-3 and siRNA-4 compared to untransfected cells or cells transfected with siRNA-1 (Fig. 6b). Transfection with siRNA-2, siRNA-3 and siRNA-4 decreased cell migration on TNfn3RAA of cells expressing wild type α9 to the level seen for cells transfected with the α9α5 chimera, but had no effect on migration of cells expressing α9α4 or α9α5 (Fig. 6c). The enhancement of α9-dependent cell migration by BE-3-3-3 was also decreased by siRNA transfection (Fig. 6d). None of these treatments had any effect on cell adhesion on TNfn3RAA or on cell migration on fibronectin in any cell type (data not shown). These results further support the concept that endogenous levels of SSAT play an important role in α9-dependent enhancement of cell migration.

The target sequences of the functional siRNA-3 and siRNA-4 are the same in mouse and hamster SSAT cDNA. We therefore used these two siRNAs to inhibit the expression of endogenous SSAT in CHO cells. Both mRNA and protein concentrations of hamster SSAT were substantially reduced by each siRNA (Fig. 7a and c). However, the sequence of human SSAT is different from the mouse and hamster sequence in the regions targeted by each siRNA. Thus, neither siRNA affects the mRNA or protein concentration in cells transfected to express human SSAT (Fig. 7b and c). In CHO cells only expressing endogenous hamster SSAT, the enhancement of α9-dependent cell migration by BE-3-3-3 was decreased by siRNA transfection (Fig. 7d), just as it was in MEFs. However, α9-dependent enhancement of cell migration was not inhibited by siRNA targeting mouse (and hamster) SSAT in CHO cells over-expressing human SSAT (Fig. 7d). This reconstitution of α9-dependent enhancement of cell migration in siRNA-treated
CHO cells with human SSAT that is resistant to these siRNAs suggests that the effects of the siRNAs tested are likely specifically due to the knockdown of SSAT.

To further investigate the important of SSAT for endogenous $\alpha 9\beta 1$-dependent enhancement of cell migration in untransfected cells. Human microvascular endothelial cells (HMVEC) were used. The expression of integrin $\alpha 9\beta 1$ on the HMVEC cell surface was tested by flow cytometry (Fig. 8a). The expression of SSAT was induced in these cells by BE-3-3-3 and inhibited by siRNA directed against human SSAT. Two siRNAs were evaluated, and only one (siRNA-A) consistently inhibited SSAT mRNA concentrations in untreated cells (Fig. 8b) and dramatically reduced SSAT protein levels in BE-3-3-3 treated cells (Fig. 8c). Induction of SSAT increased HMVEC migration on the $\alpha 9\beta 1$- specific ligand, TNfn3RAA. Inhibition of SSAT by functional siRNA specifically decreased cell migration on Tnfn3RAA (Fig. 8d). Neither BE-3-3-3 nor siRNA against human SSAT had any effect on cell migration on the $\alpha 9\beta 1$ irrelevant ligand, plasma fibronectin (Fig. 8e). These results demonstrate that the expression level of SSAT specifically affects $\alpha 9\beta 1$-dependent cell migration in cells that endogenously express $\alpha 9\beta 1$.

**Discussion**

We have previously demonstrated that the integrin $\alpha 9\beta 1$ mediates enhancement of cell migration (Taooka et al., 1999; Young et al., 2001). As with the closely related integrin, $\alpha 4\beta 1$, this effect depends on sequences within the $\alpha$ subunit cytoplasmic domain. However, in contrast to the mechanism used by $\alpha 4$, $\alpha 9$-dependent enhancement
of migration does not require the adaptor protein, paxillin (Young et al., 2001). We now show that the acetyltransferase, SSAT, specifically and directly binds to the α9 cytoplasmic domain, that increased levels of SSAT further accelerate cell migration only in cells expressing wild type α9 and only on α9β1-specific substrate, and that decreased levels of SSAT only decrease cell migration under the same circumstances. Furthermore this effect of SSAT appears to require catalytic activity. Together, these results strongly suggest that catalytically active SSAT is a critical component of the pathway by which the integrin α9β1 enhances cell migration and that this pathway is highly specific for this integrin.

As we have previously reported, α9β1 (containing the complete α9 cytoplasmic domain) is diffusely expressed on the cell surface and does not localize to focal adhesion structures (Young et al., 2001), even in cells plated on specific ligands (e.g. TNfm3RAA). Although we are unable to detect endogenous SSAT with available reagents, heterologously expressed, Myc-tagged or RFP-tagged SSAT is diffusely expressed throughout the cytoplasm and nucleus (data not shown). Thus, while both proteins can be seen along the cell membrane we have not been able to infer “co-localization” from immunostaining experiments. Nonetheless, the broad expression pattern of each protein at least suggests that it is feasible for them to make contact in intact cells. In vivo, SSAT is ubiquitously expressed, whereas α9β1 expression is restricted to subsets of epithelial cells, muscle cells (Palmer et al., 1993), leukocytes (Taooka et al., 1999) and endothelial cells. Thus, SSAT is clearly expressed in cells that do not express α9β1, but all α9β1-expressing cells are likely to express SSAT.

Over the past several years, much has been learned about the common pathways by
which ligated integrins activate cytoplasmic signals (outside-in signaling) and by which cytoplasmic signals affect the affinity and avidity of integrins (inside-out signaling). However, the divergent phenotypes of mice expressing null mutations of individual integrin subunits and the divergent effects of specific integrins on cell behavior make it clear that the signaling pathways utilized by integrins can be highly specific. In this paper, we describe a novel example of integrin specificity that appears to be critical for the best-characterized cellular function of the α9β1 integrin, acceleration of cell migration.

The oxidative catabolism of the higher polyamines spermidine and spermine is accomplished by the concerted action of two different enzymes, SSAT and polyamine oxidase (PAO). Cytosolic SSAT N\(^1\) acetylates both spermidine and spermine which then serve as substrates for peroxisomal PAO (Holtta, 1977). As PAO strongly prefers acetylated polyamines to the unmodified polyamines as its substrates, SSAT is generally considered as the rate-controlling enzyme in the back-conversion of the higher order polyamines, spermidine and spermine to the lower order polyamine, putrescine (Casero and Pegg, 1993). Thus far, the higher order polyamines, spermine and spermidine are the only physiological substrates that have been identified for SSAT. It is therefore tempting to speculate that SSAT enhances α9β1-dependent cell migration by local effects on polyamines. Indeed, previous studies have implicated polyamines in regulation of cell migration, although the precise mechanisms of action of polyamines in this process have been obscure (Johnson et al., 2002; Ray et al., 2003). Depletion of polyamines by treatment with DFMO, a drug that blocks the enzyme ODC responsible for conversion of ornithine to putrescine, has been shown to globally inhibit cell migration and also reduce cellular activity of the small GTPase rac1, a well-established mediator of cell migration.
(Johnson et al., 2002; Ray et al., 2003). It is thus conceivable that SSAT-mediated modulation of local polyamine concentrations enhances local rac1 activity to enhance cell migration. However, an equally plausible hypothesis is that SSAT is involved in acetylation of other unknown substrates whose acetylation affects their role in enhancing cell migration.

The α9β1 integrin is expressed on a number of cells for which migration is critical for function. For example, this integrin is expressed on neutrophils and monocytes and has been shown previously to play an important role in neutrophil emigration across activated endothelial cells (Takouka et al., 1999). Transendothelial migration appears to depend on binding of α9β1 to VCAM-1, an inducible ligand expressed on endothelial cells at sites of infection, tissue injury and inflammation. Over the past several years, a number of other α9β1 ligands have been identified, and many of them, including tenascin C (Yokosaki et al., 1994), osteopontin (Smith et al., 1996; Yokosaki et al., 1999), tissue fibronectin (Liao et al., 2002), and the coagulation proteins von Willebrand factor and factor XIII (Takahashi et al., 2000) are highly enriched in injured and inflamed tissues. Thus, α9β1 is likely to play an important role both in leukocyte emigration out of the vasculature and in leukocyte migration through the extravascular space at sites of injury, infection or chronic inflammation. Furthermore, mice expressing a null mutation of the α9 subunit demonstrate defects in development of lymphatic vessels (Huang et al., 2000), suggesting a possible role for α9β1 in the migration of lymphatic endothelial cells required for normal lymphatic development. Our identification of SSAT as a critical effector molecule in α9β1-dependent cell migration thus provides a new target for intervention in disorders characterized by excessive leukocyte emigration and for
modulation of migration of other α9β1-expressing cells.

METHODS

Reagents

The α9β1-specific ligand used in this study was a recombinant form of the third fibronectin type III repeat of chicken tenascin-C (Prieto et al., 1993) containing alanine (A) substitutions for both glycine (G) and aspartate (D) residues within the arginine (R)-G-D site (TNfn3RAA) (Yokosaki et al., 1998a; Yokosaki et al., 1994). Mouse mAb, Y9A2, raised against human α9β1 (Wang et al., 1996) were prepared as previously described. Monoclonal antibody 9B11 against the Myc-Tag was purchased from Cell Signaling Technology (Beverly, MA). N^1,N^11-bis(ethyl)norspermine tetrahydrochloride (BE-3-3-3) was purchased from Tocris (Ellisville, MO). Polyclonal antibody against integrin β1 was purchased from Chemicon International (Temecula, CA). Anti-Crk was purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Polyclonal anti-SSAT antibody was prepared as described previously (Casero et al., 1991).

Yeast two-hybrid screening

The yeast Matchmaker two-hybrid system (CLONTECH Laboratory, Palo Alto, CA) was used for a library screen. In brief, α9, α2, α4, α5 and β1 cytoplasmic domains (aa 2883-3053 for α9) were amplified by PCR and cloned into the Gal4-DNA binding domain (Gal4-DB) vector (pAS2-1) to screen a library of human leukocyte cDNAs fused to the Gal4-DNA activation domain (Gal4-DA) vector pACT2. AH109 were used as the
host strain. Yeast two-hybrid library screening was performed according to the Matchmaker manual on plates lacking adenine, histidine, leucine and tryptophan. Positive interactions were confirmed by β galactosidase expression on X-α-Gal.

**Expression and Purification of GST-fusion Proteins**

GST-fusion proteins containing the α9, α2, α4 and α5 cytoplasmic domains were generated by cloning the PCR-amplified sequences into the bacterial expression plasmid and expression in BL21-Gold bacteria (Stratagene, San Diego, CA). Transformed cells were cultured in 300 ml of YT medium in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma, St. Louis, MO) and GST fusion proteins were purified on glutathione-Sepharose by B-PER GST Fusion Protein Purification Kit (Pierce Biotechnology, Rockford, IL).

**In Vitro Transcription and Translation and protein binding assays**

*In vitro* transcription and translation experiments were done with the T7 RNA polymerase transcription/translation systems using rabbit reticulocyte lysate (Promega, Madison, WI) and L-[35S]methionine (Amersham Pharmacia Biotech, AG1094) to produce 35S-labeled proteins according to the manufacturer's recommendations. Plasmids containing full-length, truncated, or mutant forms of SSAT in pBluescript SK were used as templates. 0.8 nmol of the appropriate GST fusion protein was mixed with 40 µl of the *in vitro* translation reaction above and 50 µl of glutathione-Sepharose beads, incubated in binding buffer (phosphate-buffered saline with 1% Triton X-100) at 4°C for 2 h. The beads were washed 5 times in 1 ml of binding buffer and re-suspended in 25 µl
Laemmli sample buffer and heated at 95°C for 5 min. Bound proteins were separated by SDS-PAGE (15%) under reducing conditions. Gels were fixed in 50% methanol, 10% acetic acid for 30 min, soaked in a fluorographic reagent, Amplify (Amersham Pharmacia Biotech) dried and developed by autoradiography.

**Generation of stable cell lines**

Integrin α9, α9α4 and α9α5 expressing CHO cells and MEFs were generated in our lab previously (Young et al., 2001). Transfected cells were analyzed for expression of α9, α9α4 and α9α5 integrins by flow cytometry with the anti-α9β1 antibody, Y9A2. Fluorescence-activated cell sorting was performed to isolate heterogeneous populations of cells expressing high levels of α9β1 integrin on their cell surface (Yokosaki et al., 1998b). All cell lines continuously expressed high surface levels of α9β1 as determined by flow cytometry with Y9A2.

Full-length SSAT cDNAs were amplified by forward primer: GCAGCGGATCCCC-GCCATGGCTAAATTCGTGATCCGCCCAGCCACTGCCGCGACT and reverse primer: CGTACCTCGAGTCAATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCCTCCTCTGTTGCCATTTTTTA to add a c-Myc tag to the COOH terminus using either wild type SSAT or the double mutant (R101A/E152K) in pblue script SK as template (Coleman et al., 1995). The PCR products were digested by EcoRI and XhoI and cloned into pcDNA3.1/Hygro(+) (Invitrogen, San Diego, CA.). Expression constructs were confirmed by restriction digestion and sequencing. Integrin α9, α9α4, and α9α5 expressing CHO cells were used for transfection. Stable clones were obtained by dilution subcloning and characterized by western blotting. These double transfectants were
maintained in medium containing 250 μg of G418/ml and 50 μg of hygromycin/ml.

Co-immunoprecipitation and western blot analysis

CHO cell lines expressing different chimeric α9β1 integrins were lysed on ice for 30 min in an immunoprecipitation buffer: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM benzamidine HCl, 0.02% sodium azide, 1% Triton X-100, 0.05% Tween 20, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Lysates were clarified by centrifuging at 16,000 g for 20 min at 4°C and then incubated with protein G-Sepharose coated with the anti-α9β1 antibody, Y9A2 at 4°C overnight. 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 and probed with anti-Myc monoclonal antibody and detected by ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Cell surface protein biotinylation and co-immunoprecipitation:

To determine whether there is interaction between SSAT and cell surface α9β1, labeling of surface proteins was performed using the lysine-directed, membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL). Cells were washed 4 times with PBS at 4°C and then incubated with 1.5 mg/ml sulfo-NHS-SS-biotin in PBS for 30 min at 4°C. After the sulfo-NHS-SS-biotin incubation, cells were washed twice with 100 mM glycine in PBS and then incubated for 20 min at 4°C in glycine/PBS. The glycine buffer was removed and lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.05% Tween 20,
1×protease inhibitor cocktail) applied, followed by shaking for 30 min at 4°C. The supernatants were cleared of insoluble material by pelleting at 15,000 rpm for 20 min at 4°C. ImmunoPure immobilized streptavidin beads (Pierce Biotechnology, Rockford, IL) were washed 3 times with 100 mM glycine/PBS and then 4 times with the lysis buffer, finally resuspending the beads in lysis buffer. 210 µl of bead/lysis buffer slurry was added to 750 µl of supernatant and gently mixed for 1 h at room temperature. Following incubation, the streptavidin beads were washed 4 times with lysis buffer, and biotinylated proteins were eluted from the beads with lysis buffer containing 50 mM DTT. Eluted samples were used for co-immunoprecipitation as described above.

**Small interfering RNA construction and transfection**

Small interfering RNAs (siRNAs) to mouse SSAT were designed with 3′-overhanging thymidine dimers as described (Elbashir et al., 2001). Target sequences were aligned to the mouse genome database in a BLAST search (www.ncbi.nlm.nih.gov/blast) to eliminate those with significant similarity to other genes. Web-based siRNA design software from Ambion (www.ambion.com/techlib/misc/siRNA_finder.html) was used for selecting siRNA sequences. Four siRNA (siRNA-1: UCUAAGCCAGGUUGCCAUGTT; siRNA-2: AAGAAGAGGUGCUUCGGAUTT; siRNA-3: CACCCCUUCUACCACUGGCTT; siRNA-4: AAAUGGCAGCAGAGGAGUGTT) for mouse SSAT and two siRNA (siRNA-A: UGGCUAAAUUGUGAUCCGTT; siRNA-B: GAUGGUUUUGGAGAGCACCTT) for human SSAT were synthesized (Proligo USA Corp, La Jolla, CA) and used for transfection with the siPORT™ Amine Transfection Agent (Ambion, Inc., Austin, TX).
In brief, MEF cells or HMVEC were grown to 50-70% confluence in complete medium without antibiotics in 6-well plates. Cells were washed with serum-free medium. 10 μl SiPORT Amine was added into Opti-MEM I medium for a final complexing volume of 200 μl, vortexed and incubated at room temperature for 10–30 min. 1.25 μl of 20 μM siRNA was added, mixed gently and incubated for 15 min. This mixture was added dropwise to cells in a volume of 800 μl of Opti-MEM I.

Flow cytometry

Cultured cells were harvested by trypsinization and rinsed with phosphate-buffered saline (PBS). Nonspecific binding was blocked with normal goat serum at 4°C for 10 min. Cells were then incubated with primary antibody (Y9A2) for 20 min at 4°C, followed by a secondary goat anti-mouse antibody conjugated with phycoerythrin (Chemicon, Temecula, CA). Between incubations cells were washed twice with PBS. The stained cells were re-suspended in 100 μl of PBS, and fluorescence was quantified on 5000 cells with a FACScan flow cytometer (Becton Dickinson, Rutherford, NJ).

Cell adhesion assays

The wells of non-tissue culture treated 96-well microtiter plates (Nunc, Naperville, IL) were coated by incubation with 100 μl of TNfn3RAA for 1 h at 37°C. After incubation, wells were washed with PBS and then blocked with 1% bovine serum albumin (BSA) in DMEM at 37°C for 30 min. Control wells were filled with 1% BSA in DMEM. The cells were detached with 2.5 ml of trypsin solution (Sigma, St. Louis, MO), followed by 2.5 ml of trypsin-neutralizing solution (Sigma, St. Louis, MO), washed once in DMEM, and
re-suspended in DMEM at 5 ×10^5 cells/ml in the presence or absence of 50 µg/ml Y9A2 for 20 min at 4°C before plating. Plates were centrifuged (top side up) at 10×g for 5 min before starting the incubation for 1 h at 37°C in humidified 5% CO_2. Non-adherent cells were then removed by centrifugation (top side down) at 48×g for 5 min. Attached cells were fixed and stained in 40 µl of a 1% formaldehyde, 0.5% crystal violet, 20% methanol solution for 30 min, after which the wells were washed three times with PBS. The relative number of cells in each well was evaluated after solubilization in 40 µl of 2% Triton X-100 by measuring the absorbance at 595 nm in a microplate reader (Bio-Rad, San Francisco, CA). All determinations were carried out in triplicate, and the data represent the means ±SEM for a minimum of three experiments.

**Cell migration assays**

For chemotactic migration assays, 24-well Transwell plates (Costar, Cambridge, MA) were used. The lower side of the Transwell filters (6.5-mm diameter, pore size 8.0 µm) were coated with TNfn3RAA dissolved in 250 µl of DMEM for 60 min at 37°C. After incubation with TNfn3RAA, filters were washed by adding 100 µl of PBS to the top well and 500 µl of PBS to the bottom well. After washing twice, filters were blocked with 1% BSA in DMEM for 30 min and again washed once in PBS. Cells were detached as described above and resuspended in DMEM at 5×10^5 cells/ml. Migration and adhesion assays were performed at the same time, and the cells from the same dishes were used for both assays. Cells were incubated for 20 min on ice with or without the anti-α9β1 antibody, Y9A2 (50 µg/ml), and then 100 µl were loaded (50,000 cells/chamber) in each chamber. Each chamber was inserted into a well containing 600 µl of DMEM
supplemented with 1% FCS to serve as a chemoattractant and incubated at 37°C in humidified 5% CO₂ for 2 h for the CHO cells or 3 h for the MEF cells. Medium was then aspirated and the filters washed once with PBS. Cells on the bottom of the filters were fixed for 20 min in 500 µl of DifQuik fixative (Fisher, Springfield, NJ), and the nonmigrated cells on the top of the filter were gently removed. Filters were allowed to completely dry, stained by DifQuik, washed in running distilled H₂O and allowed to destain in distilled H₂O for 1 h. Filters were air-dried (≥3 h), removed from the chamber with a scalpel, and mounted onto glass slides with the use of a Permount/xylene solution, and the migrated cells were counted. Migrated cells were counted under a 25× objective with the use of a gridded eyepiece (reticule). Ten high-powered fields (HPF) per slide were counted, the average was taken, and the number of migrated cells was expressed as migrated cells per 10 HPF. The data represent means ±SEM from a minimum of three experiments.

**Scratch wound assay**

Tissue culture dishes were coated with 5 µg/ml GST-TNfn3RAA overnight at 4°C. Cells were suspended with trypsin/EDTA and plated onto coated dishes at a density of 10⁶ cells/35mm dish. After 2 h, cell cultures were scratched with a single pass of a pipet tip, and incubated at 37°C for 8 h. In some cases, culture media was supplemented with BE-3-3-3 (50µM). Cultures were washed twice with PBS and fixed in 3.7% formaldehyde for 10 min and photographed. Cells were viewed on a Nikon inverted microscope (PE300, Nikon, Tokyo, Japan) using Nikon objective lens (10×/ 0.30 20×/ 0.45). Images were processed by Openlab 2.2.5 software (Improvision, Coventry, UK)
and Hamamatsu ICCD digital camera (C4742-95, Hamamatsu Photonics, Hamamatsu city, Japan) at room temperature and arranged and labeled using Adobe Photoshop.

**ABBREVIATIONS**

SSAT: spermidine/spermine N1-acetyltransferase; siRNA, small interfering RNA; PBS: phosphate buffered saline; MEF: mouse embryonic fibroblast; Tnfn3RAA, an RAA for RGD mutant fragment of the third fibronectin type III repeat of tenascin-C; SDS-PAGE: SDS-polyacrylimide gel electrophoresis, CHO: Chinese hamster ovary; BE-3-3-3: \(N^3,N^{11}\)-Diethylnorspermine tetrahydrochloride

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**Figure 1. SSAT specifically binds to the α9 cytoplasmic domain**

(a) Plasmid DNA isolated from positive colonies from α9 yeast two-hybrid library screening were co-transformed into yeast with pAS2-1 containing integrin α2, α4, α5, α9 or β1 cytoplasmic domain cDNA. At 72 hours following the transformation, yeast growth on YPD (-Ade/-His/-Leu/-Trp/X-α-gal) plate were shown. (b) GST pull-down assays with the α9, α2, α4 and α5 cytoplasmic domain fused to GST and immobilized on Glutathione Sepharose 4B. Wild type SSAT protein was produced by in vitro transcription and translation (IVTT) in the presence of [35S]methionine. Bound proteins were separated by 15% SDS-PAGE and analyzed by autoradiography. 25% of IVTT products were also analyzed to show the same amount of input. (c) Wild type and full-length SSAT, N terminal deletion SSAT (21-171), C terminal deletion mutants (1-161), (1-141) and enzymatically inactive mutant SSAT (R101A/E152K) proteins were produced by IVTT, pulled down by GST-α9 and analyzed as above.

**Figure 2. Co-immunoprecipitation of α9β1 and SSAT.**

(a) Flow cytometric evaluation of cell surface expression of α9 integrin on α9-, α9α5 and α9α4-CHO cells. Open peaks represent fluorescence (FL) of unstained CHO cells, and shaded peaks represent fluorescence of CHO stained with the anti-α9β1 antibody, Y9A2. (b) CHO cells transfected with Myc-tagged SSAT were treated by BE-3-3-3 (50 µM) and immuno-precipitated by protein G-Sepharose coated with the anti-α9β1
antibody, Y9A2. Precipitated polypeptides were separated by SDS-PAGE under reducing conditions and detected with anti-Myc monoclonal antibody or anti-integrin β1 cytoplasmic domain anti-serum.

**Figure 3. Effects of SSAT induction by BE-3-3-3 on adhesion and migration of α9-expressing CHO cells.**

(a) CHO cells were treated by BE-3-3-3. SSAT was detected by western blotting with rabbit polyclonal anti-SSAT. (b) α9-, α9α4- and α9α5-expressing CHO cells were treated with a range of concentration of BE-3-3-3 for 24 hours and were then suspended in serum-free medium and seeded onto membranes coated with 5 µg/ml TNfn3RAA in the upper well of 24-well plates in the presence or absence of the anti-α9β1 mAb, Y9A2. After a 2 h incubation in the presence of 1% FCS in the bottom well, non-migrated cells on the top side of the membrane were removed, and migrated cells on the bottom side were fixed, stained, and counted. (c) As above, cells were suspended in serum-free medium and added to 96-well plates coated with 5 µg/ml TNfn3RAA in the presence or absence of the anti-α9β1 mAb, Y9A2. Cells were allowed to attach for 60 min, and non-adherent cells were removed by centrifugation. Adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595nm. (d) Migration of α9-, α9α4- and α9α5-expressing CHO cells was analyzed on plasma fibronectin (5 µg/ml). All data represent the means (±SEM) of triplicate experiments.

**Figure 4. The induction of SSAT by BE333 enhances α9 mediated cell migration.**

(A) CHO cells stably expressing wild type α9 or chimera α9α5 were plated onto dishes
coated with 5 µg/ml TNfn3RAA, scratch wounded at confluence, and allowed to migration into the wound space for 8 h. The ratio of the final wound area to the area immediately after scratching is indicated as percent closure (B) Cells transfected with wild type α9 were incubated with or without BE-3-3-3 and fixed 8 h after scratch wounding.

**Figure 5. Adhesion and migration of α9-expressing CHO cells transfected by SSAT**

α9-expressing CHO were transfected with empty plasmid (mock), wild type SSAT (WT) or enzymatically inactive mutant SSAT (DN). Cells were treated with or without 50 µM BE-3-3-3 for 24 hours. (a) Cell migration on TNFN3RAA (5 µg/ml), (b) cell adhesion on TNFN3RAA (5 µg/ml) and (c) cell migration on fibronectin (5 µg/ml) were assayed as described for Figure 3. All data represent the means (±SEM) of triplicate experiments. (d) Western blot showing level of SSAT protein detection in mock- and SSAT-transfected α9-expressing CHO cells in the presence or absence of treatment with BE-3-3-3.

**Figure 6. Effects of siRNA knockdown on α9-dependent enhancement of cell migration**

(a)α9-expressing MEFs were transfected by siRNA-1, siRNA-2, siRNA-3, siRNA-4 and no siRNA control in 6-well plates. 24 h later, SSAT mRNA was assayed by quantitative PCR and expressed as % basal level (no siRNA). (b) BE-3-3-3 (50 µM) was used to induce the expression of SSAT 24 h after siRNA transfection for 24 h. SSAT protein was detected by Western blot. Western blot for Crk was used as a control for equal protein loading. (c,d) α9, α9α4, α9α5-expressing MEFs were transfected by siRNA-1, siRNA-2,
siRNA-3, siRNA-4 and no siRNA and 24h later cells suspended in serum-free medium were used for migration assays (c) without BE-3-3-3, (d) with BE-3-3-3 (50 µM)

**Figure 7. Rescue of siRNA knockdown of hamster SSAT by over-expression of human wild type SSAT cDNA in CHO cells.** (a) α9-expressing CHO cells stably expressing either empty vector or wild type human SSAT (SSAT-(WT)) were transfected by siRNA-1 (non-functional), siRNA-3 and siRNA-4 in 6-well plates. 24 h later, hamster SSAT mRNA was assayed by quantitative PCR and expressed as % basal level (siRNA-1). (b) Human SSAT mRNA was assayed by quantitative PCR and expressed as % basal level (siRNA-1). (c) BE-3-3-3 (50 µM) was used to induce the expression of SSAT 24 h after siRNA transfection for 24 h. SSAT protein was detected by western blot. Western blot for Crk was used as a control for equal protein loading. (d) α9 or α9α5-expressing CHO cells with or without human SSAT expression were transfected by siRNA-1, siRNA-3 and siRNA-4 and 24h later cells were suspended in serum-free medium and used for migration assays without BE-3-3-3 (0 µM) or with BE-3-3-3 (50 µM).

**Figure 8. Effects of siRNA knockdown and BE-3-3-3 on endogenous α9-dependent HMVEC cell migration**

(a) Flow cytometric evaluation of cell surface expression of α9 integrin on HMVEC cells. Open peaks represent unstained cells, and shaded peaks represent cells stained with the anti-α9β1 antibody, Y9A2. (b) HMVECs were transfected by siRNA-A, siRNA-B and no siRNA control in 6-well plates. 24 h later, SSAT mRNA was assayed by quantitative PCR
and expressed as % basal levels (no siRNA). (c) BE-3-3-3 (50 µM) was used to induce the expression of SSAT 24 h after siRNA transfection for 24 h. SSAT protein concentration was assessed by Western blot. Western blot for Crk was used as a control for equal protein loading. (d) HMVECs were transfected by siRNA-A, siRNA-B and no siRNA and 24h later, cells were treated by BE-3-3-3 (50 µM) for 24 h. After treatment with BE-3-3-3, cells were suspended in serum-free medium were used for migration assays on TNF3RAA (5 µg/ml) (d) or on fibronectin (5 µg/ml) (e). All data represent the means (±SEM) of triplicate experiments.
Figure 1
Figure 2
Figure 3

(a) BE-3-3-3(µM) 0 10 25 50

(b) Migration (cell number)

(c) Adhesion (Absorbance at 595nm)

(d) Migration (cell number)
Figure 4
Figure 5

Graph a shows the migration (cell number) of different cell types with 0µM and 50µM concentrations.

Graph b illustrates the adhesion (absorbance at 595nm) of various cell types with 0µM and 50µM concentrations.

Graph c depicts the migration (cell number) of different cell types with 0µM and 50µM concentrations.

Graph d presents a Western blot analysis showing the expression levels of SSAT and Crk under different conditions.
Figure 6

(a) Graph showing SSAT expression levels in response to different siRNAs, with a p-value of <0.01 for the siRNA-1 treatment compared to the control.

(b) Western blot image showing protein expression levels for SSAT and Crk after treatment with siRNAs.

(c) Bar graph comparing migration cell number with and without anti-α9 for different α9 isoforms.

(d) Bar graph comparing migration cell number with and without anti-α9 for different α9 isoforms.
Figure 7
Figure 8

**a**

Counts vs FL2-Height graph showing three datasets.

**b**

Bar graph showing SSAT Expression (% basal level) for No siRNA, siRNA-A, and siRNA-B.

**c**

No siRNA, siRNA-A, siRNA-B conditions with images of Blot for SSAT and Crk.

**d**

Graph showing migration (cell number) for BE-3-3-3(0μM) and BE-3-3-3(50μM) with anti-α9 conditions.

**e**

Graph showing migration (cell number) for No siRNA, siRNA-A, and siRNA-B with BE-3-3-3(0μM) and BE-3-3-3(50μM) conditions.