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Syndecan-2 cytoplasmic domain regulates colon cancer cell migration via interaction with syntenin-1

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ABSTRACT

The cell surface heparan sulfate proteoglycan, syndecan-2, is crucial for the tumorigenic activity of colon cancer cells. However, the role played by the cytoplasmic domain of the protein remains unclear. Using colon cancer cells transfected with various syndecan-2-encoding genes with deletions in the cytoplasmic domain, it was shown that syndecan-2-induced migration activity requires the EFYA sequence of the C-terminal region; deletion of these residues abolished the rise in cell migration seen when the wild-type gene was transfected and syndecan-2 interaction with syntenin-1, suggesting that syntenin-1 functioned as a cytosolic signal effector downstream from syndecan-2. Colon cancer cells transfected with the syntenin-1 gene showed increased migratory activity, whereas migration was decreased in cells in which syntenin-1 was knock-down using small inhibitory RNA. In addition, syntenin-1 expression potentiated colon cancer cell migration induced by syndecan-2, and syndecan-2-mediated cell migration was reduced when syntenin-1 expression diminished. However, syntenin-1-mediated migration enhancement was not noted in colon cancer cells transfected with a gene encoding a syndecan-2 mutant lacking the cytoplasmic domain. Furthermore, in line with the increase in cell migration, syntenin-1 mediated Rac activation stimulated by syndecan-2. Together, the data suggest that the cytoplasmic domain of syndecan-2 regulates colon cancer cell migration via interaction with syntenin-1.

1. Introduction

Syndecans, cell surface heparan sulfate proteoglycans, are known to play diverse roles in cell adhesion and to mediate adhesion-dependent functions. Since a key feature of cancer cells is a lack of cell–cell adhesion and enhanced migration, the syndecans are involved in regulation of cancer cell behavior. Of the four members of the syndecan family, syndecan-2 is conspicuously protumorigenic in various cancer cells. Syndecan-2 overexpression is significantly associated with carcinogenesis and cancer progression in cancer of the prostate [1,2] and melanoma [4,5], and in fibrosarcoma cells [3] and colon cancer cells [6]. Cell migration induced by syndecan-2 is particularly evident in the latter type of cells.

Indeed, syndecan-2 expression is increased in colon carcinoma cells and such enhanced expression is important in regulation of cell migration [6]. Syndecan-2 may control migration by acting as a docking receptor for pro-matrix metalloproteinase (MMP)-7 [7], probably via regulation of extracellular matrix turnover. Simultaneously, syndecan-2, a cell surface coreceptor, may be involved in regulation of colon cancer cell migration via a mechanism involving interaction with integrin 21 [8]. The syndecan-2-mediated increase in cell migration may be associated with alterations in migratory cell morphology, because syndecan-2 expression induces a mesenchymal-like phenotype in colon epithelial cells [9].

During cell adhesion, the ability to initiate intracellular signaling is dependent on interactions between cytosolic proteins. Several adaptor proteins are known to interact with the cytoplasmic domain of syndecan-2. These include the PDZ proteins, which provide a framework for recruitment of target molecules into membrane-bound macromolecular complexes [10]. Syntenin, a PDZ domain-containing adapter protein, originally identified as a syndecan-binding protein, is known to be involved in the organization of protein complexes in the plasma membrane. Interestingly, syntenin-1 is highly expressed in several metastatic breast cancer cell lines, including MDA-MB-435 and MCF7, and the gastric cell line Az521; syntenin-1 overexpression enhances the migratory ability of MCF7 cells [11]. Such a pro-migratory potential has been also reported in melanoma cells [12]. Therefore, it is very possible that syntenin-1 may serve as an adaptor molecule under conditions of high cell motility, such as cancer metastasis. Although syntenin-1 is known to interact with syndecan-2 [13], and both syntenin-1

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and syndecan-2 regulate cancer cell migration, no report on regulation of syndecan-2 function by syntenin-1 has appeared. Here, we report that syntenin-1 serves as a scaffolding regulator in syndecan-2-mediated colon cancer cell migration.

2. Materials and methods

2.1. Reagents and antibodies

Monoclonal antibodies (mAb) against syntenin-1 were purchased from Santa Cruz Biotechnology (CA, USA). Mouse monoclonal Rac1 antibody was purchased from Millipore (Billerica, MA). Glutathione Sepharose 4B was purchased from Amersham Bioscience (Buckinghamshire, UK). Effectene transfection reagent was purchased from Qiagen (Hilden, Germany) and LipofectAMINE 2000 reagent was purchased from Invitrogen (CA, USA). Transwell plates were purchased from Costar (Cambridge, MA). Isopropyl-β-D-thio-galactopyranoside (IPTG) was purchased from Sigma (St. Louis, MO).

2.2. Cell structure and transfection

Human colon adenocarcinoma cell line HT29 and HCT116 cells were maintained in Mccoy's 5A complete media (Welgene, Korea) and SW480 cells were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Welgene) supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml, Sigma). Transient transfections were carried out using either the LipofectAMINE 2000 reagent (Invitrogen) or the Effectene reagent (Qiagen), according to the manufacturer's protocols. RNA extraction and reverse transcription reagent according to the provided protocol.

2.3. Molecular construct

The cytoplasmic deletion mutant of syndecan-2; syndecan-2-ΔC4, -ΔC14, -ΔC31 mutants were constructed by PCR amplification following primers that were designed against the syndecan-2 mRNA of Rattus norvegicus. Forward primer was identical in three cases of mutants, described as following; 5′-TTGAATTCA TGCGGGTACGAGCCACG-3′. The reverse primers as following; ΔC4, 5′-GCTGCCTCGAGTCACTTAGTGGGTGC-3′, ΔC14, 5′-GCTGCC TCGAGTCAGGACGGTTTGCG-3′, and ΔC31, 5′-GCGGCCGCGTACAC-CAGCAGCAGGAT-3′. All mutations were inserted into pcDNA 3.1 expression vector by digestion with EcoRI/XhoI enzyme and confirmed by sequencing the constructs.

2.4. Synthesis and transfection of siRNA Constructs

To design oligonucleotides targeting human syntenin-1 and syndecan-2 mRNA for degradation, siRNA oligonucleotides were designed. Sequences of the primers are as follows: Human-syntenin-1 sense 5′-CCUGAAAUGUACACUAGCCTT-3′ and Human-syntenin-1 antisense 5′-GGCUAGUGUACAUUUCAGGTT-3′;Human-syndecan-2 sense 5′-GATCCCCTGACGATGACTACGCTTCTTTCAAGAGAACTGC-TACTGATGCGAAGATTTTTGGAAA-3′ and Human-syndecan-2 antisense 5′-AGCTTTTCCAAAAATGACGATGACTACGCTTCT TCTCT TGAAACTGCTACTGATGCGAAGAGGG-3′; Scrambled small interfering RNA (siGENOME Non-Targeting siRNA 2) was purchased from Dharmacon, Inc. (IL, USA). *In vitro* cotransfections were performed using LipofectAMINE 2000, according to the manufacturer's protocols.

2.5. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extracted from cultured cells was used as template for reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers: Rat syndecan-2 (forward) 5′-ATGC GGGTACGAGCCACGTC-3′ and (reverse) 5′-CGGGCGCAGCACTAGT-GAGG-3′; human GAPDH (forward) 5′-CCACCCATGGCAAATTC-CATGGCA-3′ and (reverse) 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. After an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s were carried out. The reaction products were analyzed in 1.5% agarose gels.

2.6. Immunoblotting

Cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4) containing a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, NJ, USA), incubated with the appropriate primary and secondary antibodies. The signals were visualized by enhanced chemiluminescence (ECL; Amersham Life science, Buckinghamshir, UK).

2.7. Transwell migration assay

Gelatin (10 μg/ml) was added to each well of a Transewell plate (Costar; 8-μm pore size), and then the membranes were allowed to dry at 25 °C for 1 h. The Transwell plates were assembled in a 24-well and the lower chambers were filled with the culture medium containing 0.1% BSA and FGF-2 (100 ng/ml). Cells (5×10^4) were added to each upper chamber, and the plates were incubated at 37 °C in 5% CO_2 for 20 h. The cells that had migrated to the lower surface of the filters were stained with 0.6% hematoxylin and 0.5% eosin and counted.

2.8. GST-pull down assays

Recombinant GST-syndecan-2 proteins were purified on glutathione–agarose beads as previously described [14]. Proteins bound on the bead were washed three times with lysis buffer, and mixed with either HCT116 total cell lysates. After incubation at 4 °C on a rotator for 2 h, the precipitated complex was eluted with SDS sample buffer, resolved by SDS–PAGE and immunoblotted with anti-syntenin-1 antibody.

2.9. GST–PAK–PBD binding assay

GST–PBD biding assay was performed essentially as described previously [15]. Briefly, the p21-binding domain of PAK1 (PBD) was expressed in *Escherichia coli* as a GST–PAK–PBD fusion protein, purified using glutathione–sepharose beads and incubated with 500 μg of cell lysates for 2 h. Bounding proteins were collected by centrifugation and suspended in SDS sample buffer. Proteins were fractionated by SDS–PAGE, transferred onto PVDF membranes, and the amounts of precipitated GTP-bound forms of Rac were estimated by Western blotting with an anti-Rac antibody.

3. Results and discussion

3.1. The syndecan-2 cytoplasmic domain is involved in regulation of cell migration

To explore the regulation of syndecan-2-mediated cell migration by the syndecan-2 cytoplasmic domain, we generated a series of mutants with deletions in that domain. Fig. 1A schematically shows four GST-fusion proteins containing either full-length syndecan-2 (SDC2) core protein or truncated cytoplasmic domains lacking the C-terminal EFYA (ΔC4), the central region and the EFYA (ΔC14), or the entire cytoplasmic domain (ΔC31). HCT116 human colon carcinoma cells were transfected with DNA encoding either SDC2 or the SDC2 mutants and migratory activity was assessed using the Transwell assay. Compared with untransfected cells, syndecan-2 expression enhanced migration of human colon cancer cells. However, deletion of four amino acid residues at the C-terminus (in the ΔC4 mutant) reduced syndecan-2-mediated cell migration. Two other deletion mutants lacking this sequence also failed to enhance cell migration (Fig. 1B). It is known that the sequence EFYA is minimally required for syntenin-1 interaction [16]. A GST pull down assay showed that syndecan-2 interacted with syntenin-1, but no tested syndecan-2 mutant did so (Fig. 1C). Together, the data suggest that the syndecan-2 cytoplasmic domain is involved in regulation of cell migration and that the C-terminal EFYA sequence is important to this end.

3.2. Syntenin-1 regulates colon cancer cell migration

Syntenin-1 promotes migration and invasion of human cancer cells such as those of melanoma, breast and gastric cancer [11,12,17–19]. To explore the role played by syntenin-1 in colon cancer cell migration, we first examined basal expression levels in various human colon carcinoma cells, by Western blotting. Relatively high levels of syntenin-1 were observed in very motile human colon carcinoma cells including those of the HT29, SW480 and HCT116 cells (Fig. 2A). To further explore the role played by syntenin-1 on colon cancer cell migration, HT29, SW480 and HCT116 cells were transfected with a gene encoding syntenin-1. Compared with vector-transfected cells, overexpression of syntenin-1 significantly enhanced migration of all colon cancer cells tested (Fig. 2B). Consistently, all colon cancer cells transfected with syntenin-1 siRNA showed a fall in cell migration (Fig. 2C).

As Rac is a well-known regulator of cell migration, acting via control of actin polymerization [20], we determined whether syndecan-1 expression affected Rac activation. Activation of Rac in HCT116 cells was determined using a Rac pull-down assay, employing the PBD domain of PAK to precipitate GTP-bound Rac from cell lysates. In line with the observed enhancement of cell migration, syntenin-1 expression increased Rac activity (Fig. 2D). Also, inhibition of Rac using a dominant-negative *Rac* gene (*Rac N17*) reduced syntenin-1-induced cell migration (Fig. 2E). Thus, as in other cancer cells, syntenin-1 appears to be involved in regulation of colon cancer cell migration.

3.3. Syntenin-1 regulates syndecan-2-mediated colon cancer cell migration

As syntenin-1 interacted with syndecan-2 to regulate colon cancer cell migration, we next investigated the effect of syntenin-1 on syndecan-2-mediated migration. As shown in Fig. 3A, syntenin-1 enhanced migration of both SW480 and HCT116 cells, but knockdown of syndecan-2 expression using siRNAs targeting syndecan-2 reduced syntenin-1-mediated cell migration. Similarly, syndecan-2-mediated colon cancer cell migration was reduced when cells were cotransfected with siRNAs targeting syntenin-1 (Fig. 3B). Consistently, expression of syntenin-1 potentiated syndecan-2-mediated colon cancer cell migration. However, this effect was not observed in cells expressing syndecan-2 deletion mutants lacking portions of the cytoplasmic domain (Fig. 3C). Syndecan-2 expression increased Rac activity in HCT116 cells, but syndecan-2 mutants lacking the C-terminal EFYA sequence failed to enhance Rac activity (Fig. 4A). In addition, syntenin-1 siRNA reduced syndecan-2-mediated Rac activity (Fig. 4B) and syntenin-1-mediated

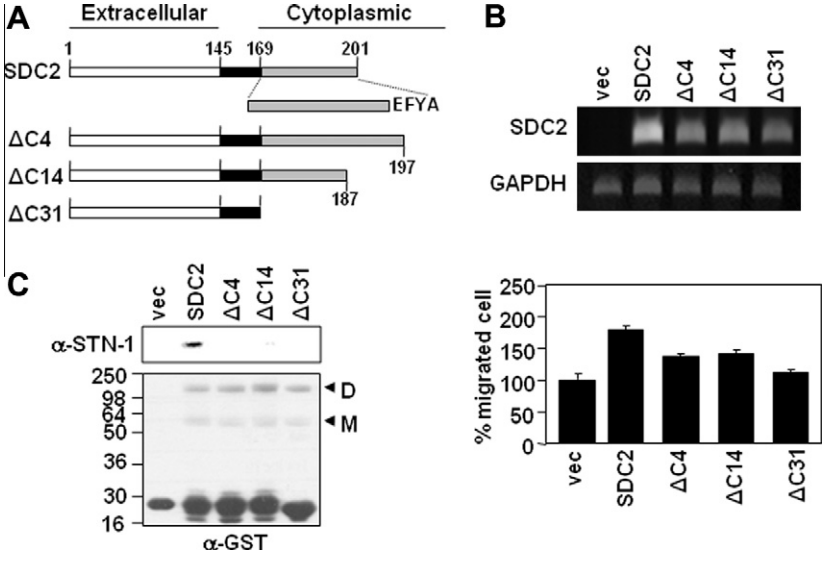

Fig. 1. The syndecan-2 cytoplasmic domain is involved in regulation of cell migration and the interaction with syntenin-1. (A) Schematic representation of GST-syndecan-2 core proteins. The extracellular domain is represented by the white box (extracellular), the transmembrane domain by the black box, and the cytoplasmic domain by the gray box (cytoplasmic). GST-fusion proteins containing full-length of syndecan-2 core protein (SDC2), truncated cytoplasmic domain mutants lacking the sequence of EFYA at the C-terminal EFYA (ΔC4), mutants lacking the central region and EFYA (ΔC14), and mutants lacking the whole cytoplasmic domain (ΔC31) are shown. (B) HCT116 cells were transfected with wild type of syndecan-2 (SDC2) or ΔC4, ΔC14, and ΔC31 mutant cDNA. Total RNA was extracted, and expression of syndecan-2 was analyzed by RT-PCR. GAPDH was used as a loading control (top panel). Cells (7.0×10^5) were allowed to migrate on gelatin-coated (10 μg/ml) Transwell plates for 20 h using FGF-2 (100 ng/ml) as a chemoattractant in the lower chamber. The results shown represent the averages of three independent experiments (bottom panel). (C) Equal amount of purified GST-SDC2, GST-ΔC4, GST-ΔC14, and GST-ΔC31 were incubated with 500 μg of total cell lysates for 2 h. Proteins bound were immunoblotted with anti-syntenin-1 antibodies (top panel). The arrows indicate the SDS-resistant dimer (D) and monomer (M) forms of syndecan-2.

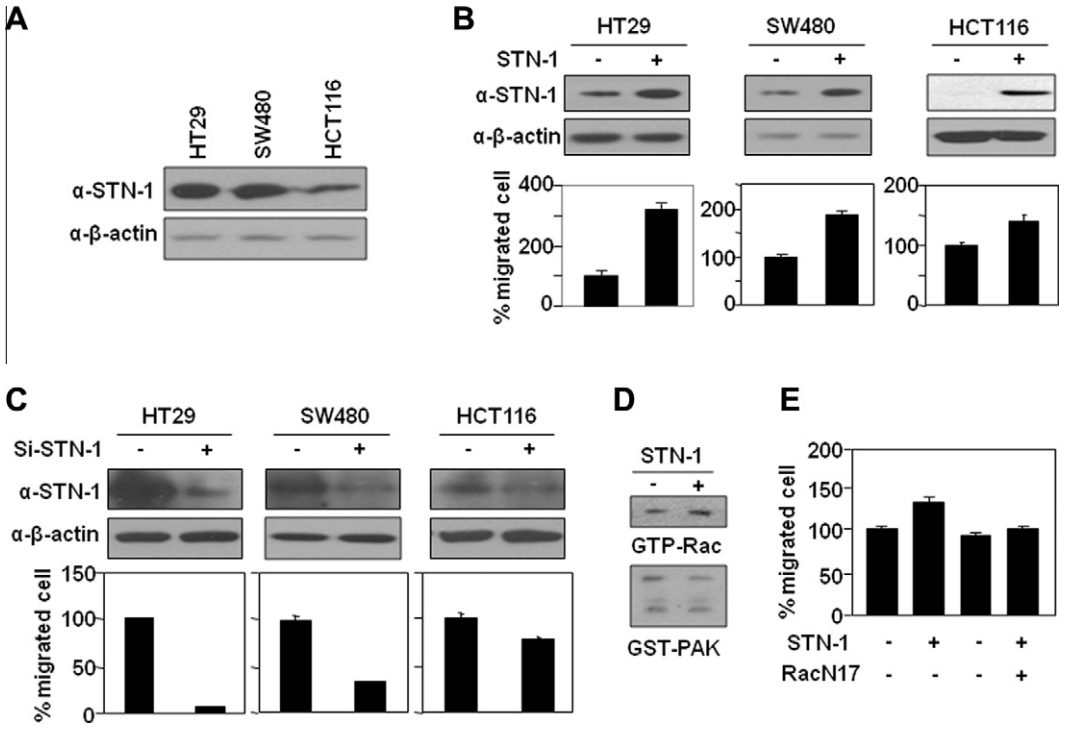

Fig. 2. Syntenin-1 regulates colon cancer cell migration. (A) Colon cancer cells indicated were lysed using RIPA lysis buffer. Total cell lysates (40 g) were resolved by SDS–PAGE and subjected to immunoblotting with anti-synteinin-1 antibody. β-actin was used as a loading control. (B) Colon cancer cells were transfected with 2 g of either empty vector or vectors encoding syntenin-1 cDNA. The expression of syntenin-1 was analyzed by immunoblotting with anti-synteinin-1 antibody. β-actin was used as a loading control (top panel). Cells were allowed to migrate on gelatin-coated (10 μg/ml) Transwell plates for 20 h using FGF-2 (100 ng/ml) as a chemoattractant in the lower chamber. The results shown represent the averages of three independent experiments (bottom panel). (C) Colon cancer cells were transfected with 100 pmol of scrambled or syntenin-1 siRNAs and Transwell migration assays were done as described in (B). (D) Purified GST–PAK–PBD fusion protein was incubated with 500 μg of cell lysates from HCT 116 cells transfected with either empty vector or vectors encoding syntenin-1 cDNA. Bounding proteins were collected and GTP-bound Rac1 was detected by Western blotting with anti-Rac antibody. Representative results from three independent experiments are shown. (E) HCT116 cells were cotransfected with syntenin-1 (STN-1) or dominant negative Rac (RacN17) cDNA. The migration assays of HCT116 cells were performed as described in (A), using gelatin (10 μg/ml)-coated transwells.

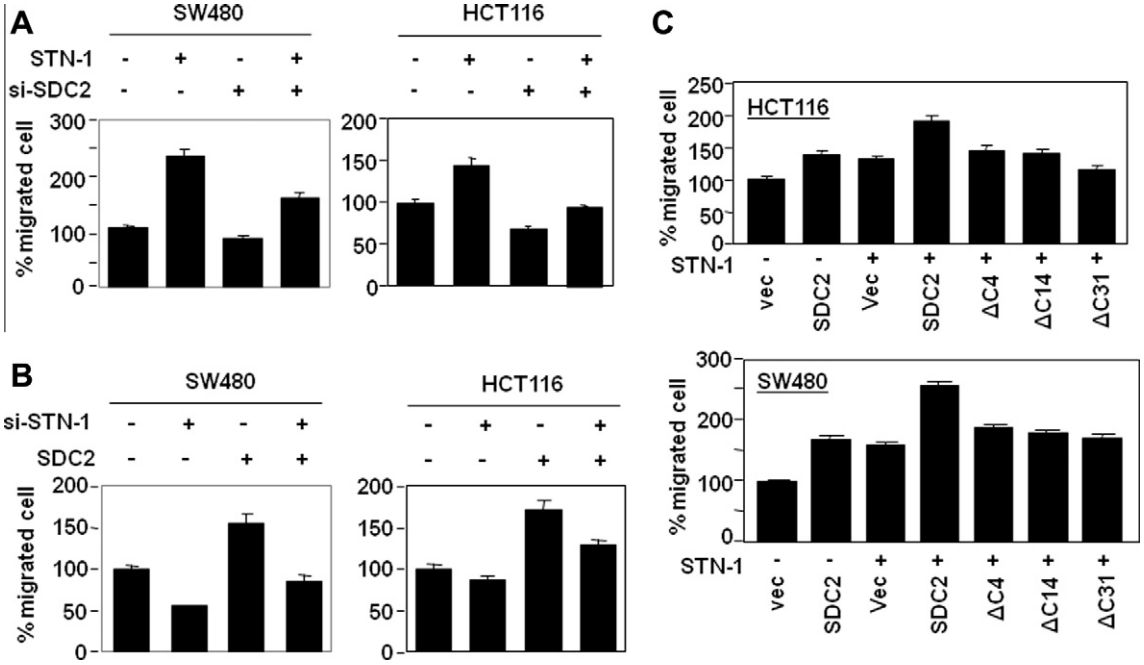

Fig. 3. Synenin-1 regulates syndecan-2-mediated colon cancer cell migration. (A) Colon cancer cells were cotransfected with syntenin-1 and either control or syndecan-2 siRNAs and allowed to migrate on Transwell plates as described in Fig. 2. The results shown represent the averages of three independent experiments. (B) Colon cancer cells were cotransfected with syndecan-2 and either control or syntenin-1 siRNAs and allowed to migrate on Transwell plates as described in Fig. 2. The results shown represent the averages of three independent experiments. (C) Either HCT116 (top panel) or SW480 (bottom panel) were cotransfected with syntenin-1 and either wild type or mutant syndecan-2 cDNA and allowed to migrate on Transwell plates. The results shown represent the averages of three independent experiments.

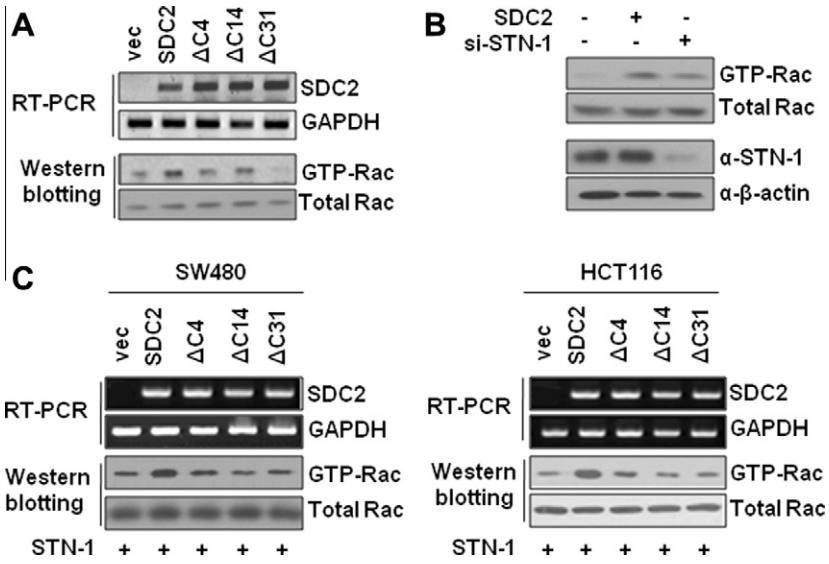

Fig. 4. Synenin-1 regulates syndecan-2-mediated Rac activation. (A) HCT116 cells were transfected with 2 g of either empty vector (vec) or vectors encoding syndecan-2 cDNA as indicated. Total RNA was extracted, and expression of syntenin-1 was analyzed by RT-PCR. GAPDH was used as a loading control (top panel). Purified GST–PAK–PBD fusion protein was incubated with cell lysates, bounding proteins were collected and GTP-bound Rac1 was detected by Western blotting with anti-Rac antibody. Representative results from three independent experiments are shown. (B) HCT116 cells were cotransfected with syndecan-2 and either control or syntenin-1 siRNAs. Expression of syntenin-1 and Rac activity assays were done as described in *A*. (C) Either SW480 or HCT116 cells were cotransfected with 2 g of syntenin-1 and syndecan-2 cDNA as indicated. Expression of syntenin-1 and syndecan-2 and Rac activity assays were done as described in (A).

potentiating of Rac activity was not observed in cells transfected with syndecan-2 mutants (Fig. 4C). Together, the data suggest that syntenin-1 regulates syndecan-2-mediated colon cancer cell migration.

The present work suggests that a full understanding of the role played by syntenin-1 may be essential for a thorough molecular dissection of syndecan-2-mediated colon cancer cell migration. Although we have shown that syntenin-1 interacts with the C-terminal EFYA sequence of syndecan-2, and thus regulates syndecan-2-mediated Rac activation and subsequent colon cancer cell migration, it remains unclear exactly how syntenin-1 stimulates syndecan-2-mediated Rac activation. It has been reported (1) that syndecan-2 regulates migration of colon cancer cells via Tiam-1-dependent Rac activation [21], (2) that syndecan-2 controls membrane localization of Tiam-1 [4], and (3) that Tiam-1 is also an important regulator of colon cancer cell migration [22]. Thus, syntenin-1 may mediate membrane localization and activation of Tiam-1. Further work is required to clarify the precise interactions between syndecan-2 and syntenin-1 that result in enhanced colon cancer cell migration.

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