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Syndecan-2 regulates cell migration in colon cancer cells through Tiam1-mediated Rac activation

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ABSTRACT

Expression of the cell surface adhesion receptor syndecan-2 is known to be involved in the regulation of cancer cell migration. However, the molecular mechanism of syndecan-2-mediated cell migration remains unknown. Here we report that Rac contributes to the regulation of syndecan-2-mediated cancer cell migration. Overexpression of syndecan-2 enhanced migration and invasion of human colon adenocarcinoma cells Caco-2 and HCT116 cells. In parallel with the increased cell migration/invasion, syndecan-2 overexpression enhanced Rac activity, while dominant negative Rac (RacN17) diminished syndecan-2-mediated increased cancer cell migration. In addition syndecan-2 expression increased membrane localization of Tiam1 and syndecan-2-mediated cell migration/invasion of Caco-2 cells was diminished when Tiam1 levels were knocked-down with small inhibitory RNAs. Furthermore, oligomerization-defective syndecan-2 mutants failed to increase membrane localization of Tiam1, activation of Rac and subsequent cell migration of both Caco-2 and HCT116 cells. Taken together, these results suggest that syndecan-2 regulates cell migration of colon carcinoma cells through Tiam1-dependent Rac activation in colon cancer cells.

Introduction

The syndecans are cell surface heparan sulfate proteoglycan that function as cell surface receptors in the regulation of adhesion-dependent signal during cell adhesion and migration [1,2] through binding of the extracellular matrix (ECM) and/or soluble ligands [3,4]. Of the four syndecan family members, syndecan-2 shows most evident as a pro-migratory receptors, not only in normal migratory cells [5,6] but also in a various number of cancer cells. Our previous studies have shown that syndecan-2 expression is increased in several cancer cell lines including colon adenocarcinoma, fibrosarcoma and melanoma, and this upregulation is crucial for migratory ability of cancer cells [7–9]. Similarly, others have shown the migratory function of syndecan-2 in Swiss 3T3 cells, colorectal cancer-derived HT29 M6 epithelial cells, and mouse brain microvascular endothelial cells [10–12]. Despite the clear indication that syndecan-2 acts as a key regulator of cancer cell migration, the molecular basis of its action remains unclear.

The initial step in cell migration is the protrusion of plasma membrane which involves localized actin polymerization. This process is regulated by Rho-like small GTPases, including Cdc42, Rac1, and RhoA [13]. In particular, it is well-known that Rac GTPases promote formation of lamellipodia in cells crawling on ECM [14]. This localized Rac activation can be achieved by focusing Rac GEF (guanine nucleotide exchange factor) at the leading edge of cancer cells. One of the ubiquitously expressed Rac-specific GEFs is Tiam1 (T-lymphoma invasion and metastasis gene1) which was first identified in a screen for genes that promote invasion in murine T lymphoma cells [15]. Tiam1 has been shown in tumorigenic cells to activate Rac1 [16,17] and Tiam1/Rac signaling regulates diverse functions including cell adhesion and migration [18]. In human colon cancer cells, knock-down of Tiam1 reduced E-cadherin-mediated adhesion in Madin–Darby canine kidney II cells [19], while overexpression of Tiam1 induced a metastatic phenotype of SW480 colon cancer cells [20], implying a positive role for Tiam1 in cancer cell migration. However, somewhat controversially, Tiam1 expression can suppress invasion probably by

Abbreviations: ECM, extracellular matrix; Tiam1, T-lymphoma invasion and metastasis gene1; GEF, guanine nucleotide exchange factor; PBS, phosphate-buffered-saline; si-RNA, small inhibitory RNA.

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promoting E-cadherin-mediated cell–cell adhesion in epithelial cells [21]. Additionally, adenocarcinomas derived from Tiam1-deficient mice are more aggressive than those from corresponding wild-type mice [22]. Although it has been well-studied that Rac plays a critical role in cancer cell migration, little is known regarding syndecan-2-mediated signaling in cancer cell migration. Here, we report that syndecan-2 regulates cell migration of colon carcinoma cells through Tiam1-dependent Rac activation in Caco-2 cells.

Materials and methods

Reagents and antibodies. Polyclonal anti-Tiam1 and anti-vinculin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody (mAb) to integrin β1 (clone 18) was from BD Pharmingen/Transduction Laboratories (San Diego, CA), and mAb to Rac1 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Effectene reagent was purchased from Qiagen (Hilden, Germany).

Cell culture and transfection. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), penicillin (1000 U/ml), and streptomycin (1 mg/ml). HT29 and HCT116 cells were maintained in McCoy's 5A medium modified Eagle's medium (Gibco BRL) supplemented with 10% FBS together with penicillin and streptomycin. Transient transfections were carried out using Effectene reagent, as described by the provided protocol.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR). Total RNA extracted from transfected cells was used as template for reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers: human syndecan-2 (forward) 5′-ACATCTCCCCTTTGCTAACGGC-3′ and (reverse) 5′-TAACTCCATCT CCTTCCCCAGG-3′; rat syndecan-2 (forward) 5′-ATGCGGGTAC-GAGCCACGTC-3′ and (reverse) 5′-CGGGAGCAGCACTAGTGAGG-3′; human GAPDH (forward) 5′-CCACCCATGGCAAATTCCATGGCA-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 55 °C for 30 s (except, GAPDH at 60 °C), and extension at 72 °C for 60 s were carried out. The reaction products were analyzed in 1.5% agarose gels.

Cell lysis and immunoblotting. The cultures were washed twice with phosphate-buffered-saline (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 13,000*g* for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Amersham Pharmacia Biotech) and probed with appropriate antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science). The signals were detected by enhanced chemiluminescence (ECL; Amersham Life Science).

Cell migration and invasion assays. For migration assays, the lower sides of transwell membranes (Costar; 8-μm pore size) were coated with gelatin B (10 μg/ml), and for invasion assays, the lower sides of the membrane were coated with gelatin (10 μg/ml), and the upper sides of the membranes with Matrigel (30 μg/μl). Then the membranes were allowed to dry at 25 °C for 1 h. The transwells were assembled in a 24-well plate, and the lower chambers were filled with the culture medium containing 0.1% BSA and bFGF. Cells (1×10^5) were added to each upper chamber, and the plate was incubated at 37 °C in 5% CO_2 for 16 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.

Rac activity assay. Rac activity assay was performed essentially as described previously [23]. Briefly, the p21-binding domain of PAK1 (PBD) was expressed in *Escherichia* coli as a GST-PAK-PBD fusion protein, and purified on glutathione-Sepharose beads (Amersham Biosciences). Each 500 μg of lysate was incubated for 2 h with purified GST-PAK fusion protein. Bound proteins were collected by centrifugation and suspended in SDS sample buffer. Proteins were fractionated by SDS–PAGE, transferred onto PVDF membranes, and GTP-bound forms of Rac associated with GST-PAK were detected by Western blotting using anti-Rac antibody.

Subcellular fractionation. After washing twice with PBS, hypo-osmotic solution (20 mM Tris/HCl, pH 7.5, 2 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA) containing a protease inhibitor cocktail was added to the culture plates. Cells were subsequently scraped off the plates, and homogenized on ice. The homogenate was centrifuged at 13,000*g* for 15 min at 4 °C to prepare the cytosolic fraction. The membrane fraction was collected by solubilizing the remaining pellet in RIPA buffer containing a protease inhibitor cocktail, and then RIPA lysates were centrifuged at 13,000*g* for 15 min at 4 °C. Equal amounts of the cytosol or the membrane fractions were resolved by 6% SDS/PAGE, transferred onto PVDF membranes and probed with an anti-Tiam1 antibody.

Results and discussion

Syndecan-2 has critical roles as an adhesion receptor during cancer cell migration [1,24]. To investigate its mechanism of action, syndecan-2 (sdc2) cDNA was transfected into human colon adenocarcinoma cells; Caco-2 cells expressing endogenously low levels of syndecan-2 and HCT116 cells expressing high levels of syndecan-2 and transwell migration assay was performed (Fig. 1A). Similar to a previous report with HT29 colon carcinoma cells [25], both cell migration and invasion were markedly increased in Caco-2 and HCT116 cells transfected with syndcan-2, compared with vector-transfected cells (Vec). Consistently, cell migration/invasion of HCT116 cells was decreased when syndecan-2 expression was suppressed by small inhibitory RNAs (siRNAs) (Fig. 1B). These results confirm that syndecan-2 functions as a pro-migratory receptor in colon cancer cells.

One important signaling cascades associated with migration/invasion is dependent on the Rho family of small GTPases [26]. Therefore, we investigated the activity of Rac using a pull-down assay, which utilizes the PBD domain of PAK to specifically isolate GTP-bound Rac from cell lysates. Expression of syndecan-2 induced increased Rac activation in all three colon cancer cells (HT29, Caco-2 and HCT116 cells), compared with controls transfected with empty vector. GTP-Rac levels were decreased in HCT116 cells transfected with sdc2 siRNA (Fig. 1C), suggesting that Rac activation is necessary for syndecan-2-mediated colon cancer cell migration. Consistently, dominant negative Rac (RacN17) diminished syndecan-2-mediated increased cancer cell migration (Fig. 1D).

Rac is activated by the exchange of GDP for GTP by GEFs, and Tiam1 is known to be a Rac-specific GEF [15]. Western blotting of whole cell lysates revealed that Tiam1 was expressed in both Caco-2 and HCT116 cells (data not shown). Interestingly, our results revealed that overexpression of syndecan-2 enhanced the membrane localization of Tiam1 (Fig. 2A), while HCT116 cells transfected with sdc2 siRNA showed decreased membrane localization of Tiam1 (Fig. 2B). These data indicate that syndecan-2 regulates the membrane localization of Tiam1 in colon cancer cells.

We next investigated whether Tiam1 was required for syndecan-2-mediated colon cancer cell migration. A 21 bp siRNA sequences targeted against human Tiam1 mRNA was employed to knock-down Tiam1 expression [8] (Fig. 3). As expected, Caco-2 cells transfected with Tiam1 siRNA showed decreased expression of Tiam1 protein

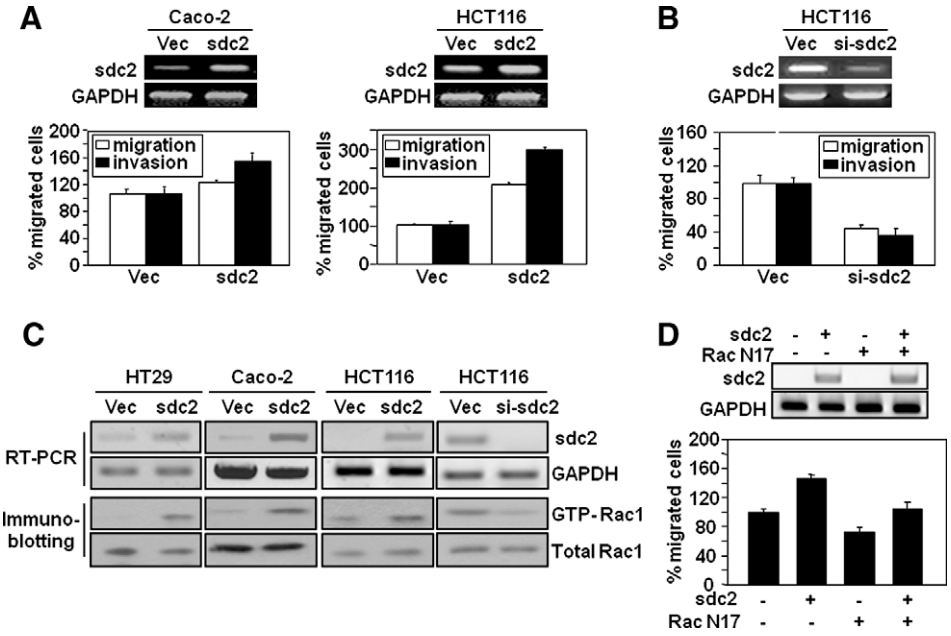

Fig. 1. Rac is involved in the regulation of syndecan-2-mediated migration of colon cancer cells. (A) Caco-2 or HCT116 human colon cancer cells were transfected with 4 μg of either vector (Vec) or syndecan-2 (sdc2) cDNA. Total RNA was extracted from these cells and mRNA expressions of sdc2 were analyzed by RT-PCR. GAPDH was used as a control (top panel). Cells (1×10^5) were allowed to migrate on either gelatin-coated (10 μg/ml) or through Matrigel-coated (30 μg/ml) transwell plates for 16 h. After fixing and staining with 0.6% hematoxylin and 0.5% eosin, the number of migrated or invasive cells was counted (bottom panel). (B) HCT116 cells transfected with either vector (Vec) or syndecan-2 siRNA (si-sdc2) were allowed to migrate on transwell plates as described in (A). The results shown represent the averages of three independent experiments. (C) Colon cancer cells were transfected with empty vector (Vec), syndecan-2 (sdc2) cDNA, or syndecan-2 siRNA as indicated. Total RNA was extracted and mRNA expressions of syndecan-2 were analyzed by RT-PCR. GAPDH was used as a control (top panel). Purified GST-PAK-PBD fusion protein coupled to glutathione agarose beads was incubated with the indicated cell lysates. Bound proteins were collected and GTP-Rac1 was detected by Western blotting with anti-Rac antibody. Total Rac in whole cell lysates is shown as a loading control (bottom panel). Representative results from three independent experiments are shown. (D) Caco-2 cells were cotransfected with syndecan-2 (sdc2) or dominant negative Rac (RacN17) cDNA. Total RNA was extracted and mRNA expression was analyzed by RT-PCR. GAPDH was used as a control (top panel). The migration assays of Caco-2 cells were performed as described in (A), using gelatin (10 μg/μl)-coated transwells (bottom panel).

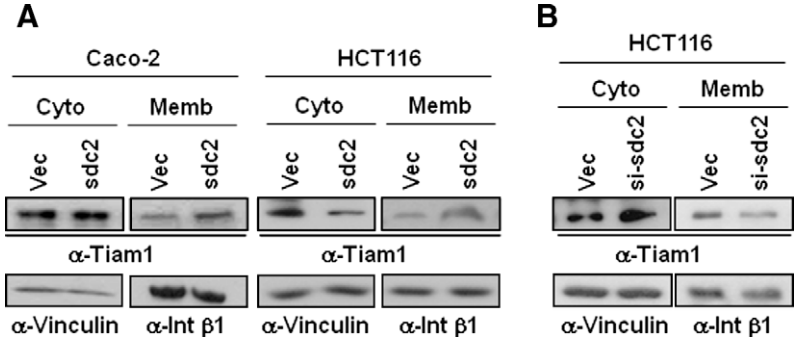

Fig. 2. Overexpression of syndecan-2 enhances the membrane localization of Tiam1. (A) Cells transfected with the indicated cDNA were lysed and fractionated, and 20 μg of protein from each fraction was resolved by SDS–PAGE and subjected to immunoblotting with antibody against Tiam1 (top panel). Anti-integrin β1 (Int β1) and anti-vinculin were used as loading controls for each fraction (bottom panel). (B) HCT116 cells transfected with either vector (Vec) or syndecan-2 siRNA (si-sdc2) were fractionated and the membrane localization of Tiam1 was analyzed as described in (A).

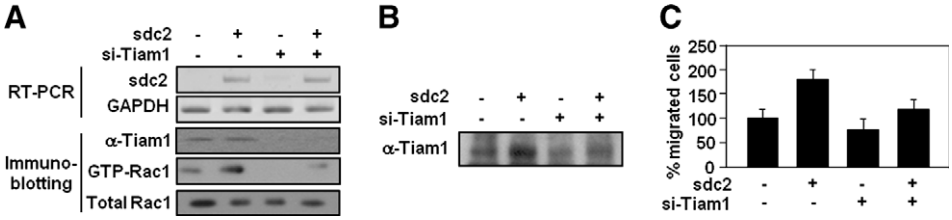

Fig. 3. Tiam1 is involved in syndecan-2-mediated migration of colon cancer cells. (A) Cells were cotransfected with syndecan-2 (sdc2) and either the pSUPER vector or Tiam1 siRNA (si-Tiam1). Cells were lysed with 1% NP-40 buffer and Rac activities were analyzed as described in Fig. 1C. (B) Caco-2 cells transfected with the indicated cDNAs were lysed and fractionated, and membrane localization of Tiam1 was analyzed as described in Fig. 2A. (C) Cells (1×10^5) were seeded on transwell plates as described in Fig. 1A. The percentages of cell migration, relative to that of vector, are shown.

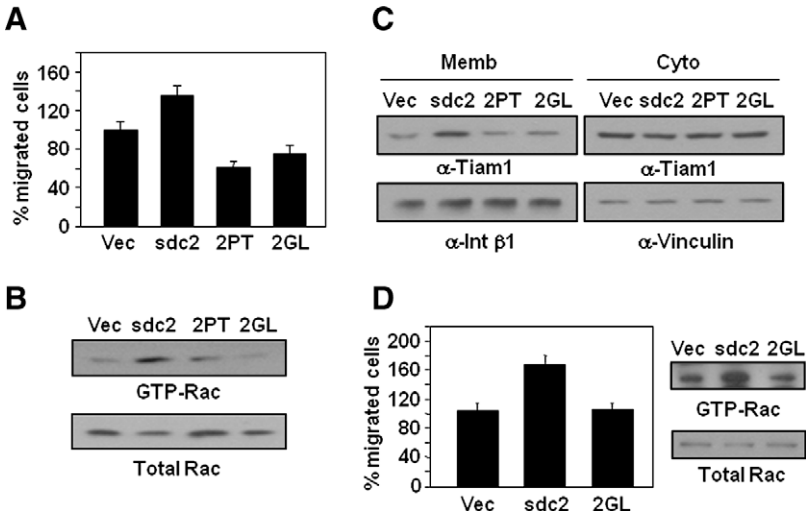

Fig. 4. Rac activation is not promoted by oligomerization-defective syndecan-2 mutants. (A) Migration assays of Caco-2 cells transfected with cDNAs of either wild type syndecan-2 (sdc2) or syndecan-2 mutants (2PT and 2GL) were performed as described in Fig. 1A, using gelatin (10 μg/μl)-coated transwells. Shown is the relative number of cell migration. (B) Cells transfected with the indicated cDNA were lysed with 1% NP-40 buffer and Rac activities were analyzed as described in Fig. 1C. (C) Cells transfected with the indicated cDNA were lysed and fractionated, and membrane localization of Tiam1 was analyzed as described in Fig. 2A. (D) The migration assays of HCT116 cells transfected with the indicated cDNAs were performed as described in Fig. 1A, using gelatin (10 μg/μl)-coated transwells (left panel). HCT116 cells were lysed with 1% NP-40 buffer and Rac activities were analyzed as described in Fig. 1C.

and concomitantly decreased Rac activity (Fig. 3A). Whereas syndecan-2 expression increased Rac activity in Caco-2 cells, cotransfection of syndecan-2 with Tiam1 siRNA reduced syndecan-2-mediated Rac activity (Fig. 3A). In addition, downregulation of Tiam1 expression reduced syndecan-2-mediated membrane localization of Tiam1 (Fig. 3B) and cell migration in Caco-2 cells (Fig. 3C). These data strongly suggest that Tiam1 regulates syndecan-2-induced cell migration/invasion in colon cancer cells.

Since syndecan-2 transmembrane domain-induced oligomerization is required for its receptor functions [27], we next investigated whether transmembrane domain-induced oligomerization might regulate the activity of Rac. Expression of syndecan-2 enhanced migration of Caco-2 cells, but both oligomerization-defective syndecan-2 mutants (2PT and 2GL) reduced cell migration (Fig. 4A). Consistently, syndecan-2 enhanced Rac activity, but oligomerization-defective syndecan-2 mutants did not show this effect (Fig. 4B). In addition, Tiam1 membrane localization was also unaffected by syndecan-2 mutant expression (Fig. 4C), supporting the importance of syndecan-2 transmembrane domain-induced oligomerization for the Rac activation. Similar results were noted in HCT116 cells (Fig. 4D). Therefore, it is likely that oligomerization of syndecan-2 is crucial for the membrane localization of Tiam1 and Rac activity to regulate syndecan-2-mediated cell migration in Caco-2 cells. Collectively, these data strongly suggest that syndecan-2 regulates cell migration of colon carcinoma cells through Tiam1-dependent Rac activation in colon cancer cells.

Previously we reported that syndecan-2 regulated cell migration/invasion of HT1080 fibrosarcoma cells through Rac dependent mechanism [8]. Consistent with its effects in HT1080 fibrosarcoma cells, overexpression of syndecan-2 increased migration of colon carcinoma cells paralleled with increased Rac activity, whereas expression of dominant Rac decreased these processes (Fig. 1). Our data indicate that syndecan-2 promotes Rac-dependent cell migration mechanism and Rac activation may be generally involved in syndecan-2-mediated cancer cell migration. We also found that syndecan-2 expression enhanced membrane localization of Tiam1 (Fig. 2) while reduced Tiam1 expression inhibited syndecan-2-mediated cancer cell migration (Fig. 3). In summary, our data indicate that syndecan-2 regulates cancer cell migration through Tiam1-dependent Rac activation. Further investigation of the cytosolic protein(s) interacting with syndecan-2 cytoplasmic domain will be necessary for clarifying detailed mechanism(s) for syndecan-2-mediated signaling.

Acknowledgments

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