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Syndecan-2 regulation of morphology in breast carcinoma cells is dependent on RhoGTPases

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ABSTRACT

Background: While syndecan-2 is usually considered a mesenchymal transmembrane proteoglycan, it can be up-regulated in some tumour cells, such as the malignant breast carcinoma cell line, MDA-MB231. Depletion of this syndecan by siRNA, but not other syndecans, has a marked effect on cell morphology, increasing spreading, microfilament bundle and focal adhesion formation, with reduced cell migration.
Methods: A combination of siRNA transfection, immunofluorescence microscopy, phosphoprotein analysis and migration assays was used to determine how syndecan-2 may influence the cytoskeleton.
Results: The altered adhesion upon syndecan-2 depletion was dependent on the RhoGTPases. p190ARhoGAP relocated to the margins of spreading cells, where it codistributed with syndecan-4 and active β_1-integrin. This was accompanied by increased RhoGAP tyrosine phosphorylation, indicative of activity and RhoGTPase suppression. Consistent with this, GTP-RhoA was strongly present at the edges of control cells, but lost after syndecan-2 reduction by siRNA treatments. Further, RhoA, but not RhoC was shown to be essential for the anchored phenotype of these breast carcinoma cells that accompanied siRNA-mediated loss of syndecan-2.
Conclusions: Syndecan-2 has a key role in promoting the invasive activity of these cells, in part by regulating the RhoGTPases.
General significance: Syndecan-2, as a cell surface receptor is accessible for targeting to determine whether breast tumour progression is altered. This article is part of a special issue, "Matrix-Mediated Cell Behavior and Properties".

1. Introduction

The syndecan proteoglycans are an evolutionarily ancient family, with four members in mammals. They are widespread, and since all can interact with the actin cytoskeleton, have been implicated in cell adhesion, migration and extracellular matrix assembly [1,2]. On their ectodomains are multiple glycosaminoglycan chains, usually heparan sulphate. This endows syndecans with an ability to interact with a large number of proteins that have heparin- or heparan sulphate-binding properties. These include many growth factors, such as fibroblast growth factors, morphogens, cytokines, chemokines, enzymes such as metalloproteinases and lipases, as well as extracellular matrix glycoproteins and collagens. With such a diverse array of binding molecules, it is apparent that numerous inputs through syndecans may lead to a conservative cytoplasmic output.

Nevertheless, specificity among syndecans has been recorded on many occasions. Syndecan-4, for example, can promote focal adhesions and its loss in the knock-out mouse leads to tissue repair and migration deficits that are not overcome by other members of the family [3,4]. In addition, syndecan-1 expression in breast cancer is associated with poor prognosis, especially where it is expressed in the tumour stroma [5,6]. The same is not true for syndecan-4 [6]. Therefore, while having common features, such as heparan sulphate chains and linkage to the cytoskeleton, there is specificity in function across the syndecans, even where multiple members of the family are expressed on the surface of a single cell type. This specificity is not well understood.

In our recent studies of the triple negative breast carcinoma cell line, MDA-MB231, lacking oestrogen receptor α, progesterone receptor and the Her2/Neu receptor, we noted the presence of syndecans-1, -2 and -4 [unpublished results]. Experiments with exogenous heparan sulphate or heparin treatment, and specific depletion of syndecans by siRNA revealed specific and important properties for syndecan-2. When depleted, these highly invasive cells spread, formed cell–cell and cell–matrix junctions, microfilament bundles and were markedly reduced in their ability to invade and degrade native type I collagen [unpublished results]. It was further shown that the microfilament bundles appeared to form in a conventional signalling pathway involving Rho kinases and elevated phosphorylation of myosin light chain [7,8]. Syndecan-2, however, is poorly understood in terms of its signalling capacities. Its cytoplasmic domain can interact with PDZ domain proteins that are probably involved in trafficking to or from the cell surface [9]. It can also interact with ezrin, but these properties are common to all syndecans [10]. Preliminary data suggest that the effect of syndecan-2

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depletion in MDA-MB231 cells was specific to this syndecan and not reproduced by reductions in either syndecan-1 or -4 [unpublished results]. As with other syndecans, association with integrins and integrin-mediated adhesion has also been recorded in various cell types, but this is not fully understood in molecular terms.

Given the striking change in behaviour of MDA-MB231 cells when depleted of syndecan-2, this proteoglycan deserves further scrutiny. While we showed an association with, and regulation of, caveolin-2 in these carcinoma cells, it remains unclear how syndecan-2 effectively suppresses the spreading and adhesion functions that are released once the proteoglycan has been depleted. Here, we determine the features of the spreading and adhesion formation, with emphasis on the RhoGTPases that are well known to influence many aspects of adhesion and locomotion.

2. Materials and methods

2.1. Cell culture

The human breast carcinoma MDA-MB231 line was maintained in Dulbecco's modified Eagle's media (Invitrogen) containing 10% foetal bovine serum (FBS) at 37 °C and 5% CO_2. Cultures were tested routinely for mycoplasma. Cells were transfected with siRNA targeting syndecan-2 (Santa Cruz Biotechnology), syndecan-4 (siGENOME SMARTpool, Thermo Scientific), p190RhoGAP A and B (siGENOME SMARTpool, Thermo Scientific), RhoA (Qiagen), RhoC (Qiagen) or non-targeting siRNA (siGENOME SMARTpool, Thermo Scientific) using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

2.2. Antibodies

The following antibodies were used: syndecan-2 (cat. H00006383-B04P; Abnova); syndecan-4 (cat. LS-C150078; LSBio), p190ARhoGAP (cat. 610150), p190BRhoGAP (cat. 611613) and p120RasGAP (cat. 610040) from BD Bioscience; RhoA-GTP (cat. 26904; Neweast Biosciences), RhoC (cat. 3430) phospho-Src (Tyr 416; cat. 2101) and phospho-(Ser) PKC substrate (P-S^3-101) (cat. 2261) from Cell Signaling; active β_1-integrin (clone HUTS-4, cat. MAB2079Z), β_1-integrin (cat. AB1592) and paxillin (clone 5H11, cat. no. 05–417) from Millipore; β-tubulin (clone TUB2.1, cat. no. T4026) from Sigma-Aldrich; Alexa Fluor-conjugated and phalloidin and Alexa Fluor-conjugated secondary antibodies used in immunofluorescence staining were obtained from Molecular Probes, Invitrogen; and peroxidase-conjugated secondary antibodies used in western blotting analysis were from Dako.

2.3. Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde/PBS and permeabilised with 0.1% Triton X-100/PBS for 10 min. This was followed by incubation in 0.1 M NH_4Cl for 20 min to quench free aldehydes and blocked with 5% heat-denatured bovine serum albumin for 30 min. Cells were incubated with indicated primary antibodies overnight at 4 °C, followed by incubation with appropriate Alexa Fluor-conjugated antibodies and/or Alexa-conjugated phalloidin for 1 h at room temperature. Coverslips were mounted with Prolong Gold mounting media (Invitrogen) and viewed on a Zeiss Axioplan-2 microscope (Carl Zeiss) using an Aprochromat 63× objective and analysed with MetaMorph software (version 6.2r6).

2.4. Western blotting, immunoprecipitation and Rho-GTPase pulldown assay

Cells were directly lysed using sample buffer before subjected to SDS–polyacrylamide gel electrophoresis and followed by electrophoretic transfer and western blotting. For p190RhoGAP immunoprecipitations, transfected cells were lysed with RIPA buffer containing 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, phosphatase inhibitors (Sigma) and protease inhibitors (Roche). The lysates were centrifuged for 5 min then precleared with protein G-agarose beads for 30 min at 4 °C before incubation with 5 μg p190ARhoGAP antibody for 1 h at 4 °C. This was followed by further incubation with protein G-agarose beads (EZview Red, Sigma) for 1 h at 4 °C before elution with sample buffer. All the samples were analysed by western blotting for phospho-tyrosine and p120RasGAP. The blots were stripped for subsequent p190ARhoGAP detection. In certain experiments, transfected cells were further treated with 10 μM of Src kinase inhibitor I (SrcI1) for 2 h before lysis. GST-Rhotekin agarose beads were prepared and Rho-GTP pulldown assays were performed as previously described [11].

2.5. Flow cytometry analysis

Transfected cells were incubated with dissociation buffer (Invitrogen), then suspended in ice-cold sterile 1% BSA/PBS and incubated with syndecan-2 antibody (1:75) or syndecan-4 antibody (1:100) for 1 h on ice. For active β_1-integrin detection, the cells were fixed with 1% paraformaldehyde/PBS for 7 min and followed by PBS washing before antibody (1:100) incubation for 1 h on ice. Incubation of appropriate Alexa Fluor-conjugated secondary antibodies for 30 min was carried out on ice. The stained cells were analysed on a FACSCalibur flow cytometer and data processed by using CellQuest Pro v6.0 software (Becton Dickinson).

2.6. Cell migration assays

The transwell inserts (12-well insert; pore size 8 μm, BD Falcon) were coated with 10 μg/ml type I collagen (PureCol™, Nutacon) and incubated for 1 h at 37 °C. Approximately 15×10^3 transfected cells were plated on the inserts in serum free medium and complete medium was placed in lower chamber as a chemoattractant. After 24 h, the cells that migrated through the filter were fixed with 4% paraformaldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted.

2.7. Statistical analyses

Error bars are presented as standard error of mean. Two-tailed paired *t*-test was used to compare between groups. $p < 0.05$ was considered significant. All statistical analysis and graphs were plotted using GraphPad Prism 6.

3. Results

3.1. Syndecan-2 regulates the distribution and activity of p190ARhoGAP to promote cell migration

The molecular mechanism by which syndecan-2 regulates cytoskeletal rearrangements is yet to be defined. However, p190RhoGAP is often involved in the regulation of contractility and is implicated in cell migration. Previous studies by Bass et al. [12] identified syndecan-4 as a regulator of p190ARhoGAP localisation in normal fibroblasts. To test whether this RhoGAP is also relevant to syndecan-2 regulated MDA-MB231 cell adhesion, we knocked down syndecan-2 levels by siRNA, which was confirmed by FACS (Fig. 1A). Control siRNA treated cells were rounded and motile in morphology while syndecan-2 depleted cells showed enhanced spreading, microfilament bundle and focal adhesion assembly (Fig. 1B). In transwell migration assays, cell migration was commensurately reduced in syndecan-2 depleted cells (Fig. 1C). In addition, p190ARhoGAP (p190A) was redistributed to the cell periphery in syndecan-2 depleted cells while no effect was noted in the localisation of p190BRhoGAP (p190B) (Fig. 1D), suggesting that syndecan-2 prevents recruitment of p190A to cell periphery where it normally exerts its activity [13,14].

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