Opposing Roles of Syndecan-1 and Syndecan-2 in Polyethyleneimine-mediated Gene Delivery*1

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Polyethyleneimines (PEIs) are efficient non-viral vectors for gene transfer. Heparan sulfate proteoglycans have been proposed to be the cell-surface receptors for PEI-DNA complexes (polyplexes). Here, we investigated if syndecan-1 (SDC1) and syndecan-2 (SDC2) are involved in PEI-mediated transfection. Following addition of polyplexes to HEK293 cells, green fluorescent protein-tagged SDCs rapidly formed clusters with PEI that were dependent of lipid raft integrity. However, although SDC1 overexpression slightly enhanced PEI-mediated gene expression, SDC2 dramatically inhibited it. Confocal microscopy analysis showed that SDC1-polyplex endocytosis occurred within minutes after addition of polyplexes, whereas SDC2-polyplex endocytosis took hours. Expression of SDC1 cytoplasmic deletion mutants revealed that the SDC1 cytoplasmic tail is required for gene expression, but not for clustering or endocytosis, whereas overexpression of SDC1/SDC2 chimeras showed that the SDC2 ectodomain is responsible for the inhibitory effect on gene transfer. This study provides evidence that SDCs may have opposing effects on PEI-mediated transfection.

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Polyethyleneimines (PEIs)2 are cationic polymers that are synthesized as either linear or branched forms and are available over a wide range of molecular masses and polydispersities. Once added to DNA, PEIs associate with and condense DNA, forming PEI-DNA nanoparticles or complexes (termed polyplexes), protecting the DNA from nuclease degradation. The capacity of polyplexes to mediate gene transfer in mammalian cells was demonstrated for the first time by Boussif et al. (1). PEIs are currently attracting great interest in the field of gene therapy because they are relatively simple to synthesize, can be linked to a wide variety of molecules for cellular targeting, and may represent a promising and safe alternative to viral vectors (2–4). In addition, they offer an inexpensive and efficient vehicle for large-scale transfection applications, enabling the rapid production of recombinant proteins and viral vectors (5). The linear and branched PEI (BPEI) 25-kDa forms have been shown to be among the most efficient non-viral vectors for in vitro (6–8) and in vivo (7–12) gene delivery. A key characteristic of PEIs resides in their intrinsic buffering capacity, a feature also known as the proton sponge effect (1). According to one hypothesis, the high buffering capacity of PEIs leads to osmotic swelling and rupture of endosomes, resulting in the release of polyplexes into the cytoplasm and passive nuclear entry during mitosis when the nuclear membrane temporarily breaks down (13).

Although it is generally accepted that membrane-associated heparan sulfate proteoglycans (HSPGs) play an important role in the uptake of polyplexes, their involvement as receptors for PEI-DNA polyplexes remains to be clearly demonstrated. The membrane-associated HSPGs encompass a wide variety of molecules, including betaglycan, also known as TGFR-3 (14); CD44v3 (15); syndecans (SDCs) (16); and glypicans (17, 18). The SDC family is composed of closely related proteins (SDC1–4) encoded by four different genes. These proteins constitute the most abundant forms of membrane HSPGs and play a central role in several aspects of cell physiology (19–22) such as cell adhesion (23), modulation of growth factor activity (24), and organization of the microfilament cytoskeleton (25). The extracellular domain of SDCs, which show little primary sequence homology, possesses three to five glycosaminoglycan attachment sites and has a putative protease cleavage site in its extracellular juxtamembrane domain. SDCs have a highly homologous transmembrane domain, which is essential for their homodimerization and oligomerization (26). The cytoplasmic domain possesses two highly homologous regions (C1 and C2) surrounding a variable region. The C2 region contains a PDZ-binding motif capable of interacting with numerous PDZ domain-containing proteins such as synemin (27), CASK/Lin-2 (28), synbindin (29), and synectin (26). Notwithstanding their predominance as cell-surface HSPGs that display a high degree of structural and functional heterogeneity, no study has been undertaken to examine the potential role of syndecans in PEI-mediated transfection.

In this study, we address the question of whether SDC1 and SDC2 could act as carriers of PEI-DNA polyplexes. We determined the impact of their overexpression on gene transfer and gene expression efficiency and analyzed their fate following addition of polyplexes. We found that although SDC1 enhances polyplex-mediated transgene expression when overexpressed in HEK293 cells, SDC2 significantly reduces it. The endocytosis, kinetics, and intracellular fate of SDC-polyplex complexes differ significantly between the two SDCs, and this may be related to their transfec-
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tion competency. Using cytoplasmic domain deletion mutants of SDC1 and SDC1/SDC2 chimeras, we evaluated the role of their various domains in polyplex-mediated endocytosis and transgene expression. Taken together, these results bring a new level of complexity to PEI-mediated transfection mechanisms and provide evidences that SDC1 may act as a receptor for polyplexes.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following antibodies were used: anti-syndecan-1 (N-18), anti-syndecan-2 (L-18), and anti-syndecan-3 (D-19) (Santa Cruz Biotechnology, Inc.) and anti-syndecan-4 (F94-8G3) (Dr. Guido David, University of Leuven, Leuven, Belgium). The following reagents were used: 293fectin™ and Lipofectin® (Invitrogen); Hoechst 33258 and rhodamine B isothiocyanate (RITC) (Sigma); PEI (Fluka); linear 25-kDa PEI and 16% ultrapure electron microscope grade formaldehyde (Polysciences, Inc.); and restriction enzymes, T4 DNA ligase, and Vent DNA polymerase (New England Biolabs).

Vector Constructions—Full-length SDC1 and SDC2, SDC1 deletion mutants, and SDC1/SDC2 chimeras were amplified and subcloned in the mammalian expression plasmid pT55 (30) in frame with green fluorescent protein (GFP) or blue fluorescent protein (BFP) (Qbiogene, Inc.) as indicated in supplemental Table 1.

PEI Labeling—BPEI was labeled with RITC by modification of a protocol described previously (31). BPEI (10 mg/ml) was mixed with 2 mg/ml RITC in 0.5 M bicarbonate buffer (pH 9.0) and incubated at room temperature under constant agitation for 2 h. The unbound RITC was removed by applying the reaction mixture to a Sephadex G-25 column (Bio-Rad).

Cell Culture and Transfection—HEK293-EBNA1 cells (clone 6E) were cultured in Freestyle™ 293 expression medium supplemented with 0.1% Pluronic™ F-68, 50 µg/ml Geneticin (G418), and 10 µM HEPES. For protein expression, 1 × 10⁶ cells were transfected using 293fectin according to the manufacturer’s instructions. Twenty-four hours post-transfection, 1 × 10⁶ cells were transfected using PEI-DNA complexes (polyplexes) made as follow. Two micrograms of PEI (for fluorescence microscopy and flow cytometry analysis) or RITC-labeled BPEI (for confocal microscopy analysis) was added to 1 µg of plasmid DNA (pT55 for fluorescence and confocal microscopy analysis or pT55-DsRed for flow cytometry analysis); both had been previously diluted in 100 µl of hybridoma serum-free medium (Invitrogen). Solutions were mixed and incubated at room temperature for 15 min before their addition to the cell culture.

Flow Cytometry Assay—The percentages of both GFP- and Discosoma sp. red fluorescent protein (DsRed)-expressing cells were determined by flow cytometry using an EPICS Profile II (Beckman Coulter) equipped with a 15-milliwatt argon ion laser. Viable transfected cells were quantified by using appropriately gating to exclude dead cells, debris, and aggregates in a forward-scatter-against-side-scatter plot.

Colocalization Experiments and Microscopy—Colocalization of fluorescent proteins and BPEI-RITC was performed using a confocal microscope. Cells were plated for 1 h onto poly-L-lysine-coated glass-bottom 35-mm culture dishes (No. 0, Mat-Tek Corp.). After washing cells with fresh medium, plated cells were transfected with BPEI-RITC for the indicated periods of time. Cells were then fixed in phosphate-buffered saline containing 4% formaldehyde for 10 min. After washing the cells twice with phosphate-buffered saline, nuclei were stained with 2 µg/ml Hoechst 33258 for 10 min.

Fluorescent images were analyzed either on a confocal microscope (Zeiss Axiovert 200M inverted microscope) using a Plan-Apochromat 63× (numerical aperture 1.4, differential interference contrast) objective or on a conventional inverted fluorescence microscope (Leica DMIL) using c-Plan 40× (numerical aperture 0.5) objective. The confocal microscope was equipped with three lasers. A laser diode (excitation, 405 nm) and a band-pass filter (420–480 nm) were used to capture the signal recorded as blue; an argon laser (excitation, 488 nm) and a band-pass filter (505–530 nm) were used to capture the signal recorded as green; and finally, a helium/neon laser (excitation, 543 nm) and a band-pass filter (550–625 nm) were used to capture the signal recorded as red. Zeiss LSM 510 META confocal system version 3.2 software and IrfanView version 3.85 were used for image acquisition by confocal microscopy and conventional microscopy, respectively.

RESULTS

Polyplexes Induce Clustering of SDC1 and SDC2 and Require Lipid Raft Integrity—Previous reports suggest that HEK293 cells express low or undetectable levels of SDC1, SDC2, or SDC4 (32–34). Accordingly, we were not able to detect SDCs by Western blotting using specific anti-SDC1–4 antibodies. However, we were able to show the presence of SDC2, SDC3, and SDC4 (but not SDC1) mRNAs by reverse transcription-PCR (data not shown).

To investigate the impact of SDC1 and SDC2 overexpression on PEI-mediated gene expression and to be able to follow their fates following polyplex addition, we tagged the C-terminal end of both proteins with GFP. The GFP-tagged truncated form of CD4 (ΔCD4), which lacks the helix in the cytoplasmic domain, preventing its internalization by Nef (35), was used as a non-HSPG control transmembrane protein. Cells were transiently transfected with each construct using the lipid-based 293fectin reagent. Twenty-four hours post-transfection (hpt), the percentage of GFP-positive cells was determined by flow cytometry. For the whole study, the average transfection efficiency using this method was 77 ± 10% (n = 37). Confocal microscopy revealed that all GFP-tagged proteins were localized mainly to the cell membrane (supplemental Fig. S1), indicating that the GFP tag does not perturb folding or targeting of these proteins.

It was reported previously that SDC4 clustering is induced in the presence of basic fibroblast growth factor-2 (36). Moreover, it has been shown that polyanine induces clustering of SDC1 (37) and that clustering of SDC2 is crucial for spine formation in neurons (38), underlying the important role of syndecan clustering for some biological activities. To determine whether PEI can induce SDC1 and SDC2 clustering, polyplexes were added to GFP-tagged SDC1- or SDC2-expressing cells, and SDCs were followed by fluorescence microscopy (Fig. 1). Within 10 min of polyplex addition, formation of GFP clusters could be easily monitored for both SDCs, but not for ΔCD4-GFP. Clustering is indeed very rapid, as it was detectable within 1 min after polyplex addition to cells. ³ Interestingly,

³ S. Paris, A. Burlacu, and Y. Durocher, unpublished data.
most of the cell surface-expressed SDCs were found concentrated in these clusters, indicating that recruitment of SDC1 and SDC2 proteins by polyplexes is very efficient. Clustering of SDC1 and SDC2 was also observed following addition of DNA-free BPEI and linear 25-kDa PEI as well as after addition of SDC1 and SDC2 proteins by polyplexes is very efficient. Clustering of most of the cell surface-expressed SDCs was found concentrated after addition of the cationic liposomes 293fectin and Lipofectin (supplemental Fig. S2).

It was reported previously that clustering and subsequent endocytosis of SDC1 and SDC4 require the integrity of plasma membrane lipid rafts (40, 41). To determine whether lipid raft integrity is also required for polyplex-induced clustering of these receptors, SDC1- and SDC2-expressing cells were treated for 1 h with 10 μM methyl-β-cyclodextrin (MβCD), a cyclic oligosaccharide known to deplete cholesterol from the plasma membrane and to disrupt lipid raft integrity. Polyplexes were added to cells, and SDC1 and SDC2 clustering was followed by fluorescence microscopy. After 10 min (Fig. 1) and even after longer periods (1 and 3 h), clustering of SDC1 and SDC2 was completely inhibited by MβCD.

**Gene Expression Is Enhanced by SDC1 but Inhibited by SDC2**—To determine the impact of SDC1 and SDC2 overexpression on PEI-mediated transgene expression, cells expressing SDC1, SDC2, or ΔCD4 were transfected with polyplexes containing a DsRed-expressing vector. At 24 hpt, the percentages of double-fluorescent cells (green for SDC and ΔCD4 expression and red for DsRed expression) were determined by flow cytometry and expressed relative to ΔCD4 control, taken as 100%. PEI-mediated transfection of SDC1-expressing cells resulted in an average increase of 12 ± 4% (n = 4) in double-labeled cells compared with control cells. Surprisingly, transfection efficiencies in SDC2-expressing cells were greatly reduced by 80 ± 3% (n = 4) compared with the ΔCD4 control.

**SDC1 but Not SDC2 Rapidly Internalizes Polyplexes**—To address this striking difference between SDC1 and SDC2, we examined the fate of polyplexes by confocal microscopy using RITC-labeled PEI (Fig. 2). In good agreement with the above results, SDC1 and SDC2 clustering could be easily monitored at the cell surface 10 min after polyplex addition (Fig. 2, a and b). In addition, these clusters colocalized with polyplexes, in accordance with the expected capacity of SDC1 and SDC2 to take up PEI-DNA complexes. At 1 hpt, most SDC1 clusters were endocytosed along with polyplexes, both appearing concentrated in an area close to the nucleus (Fig. 2a and the supplemental video). In contrast, SDC2 clusters were still located at the cell surface in tight association with polyplexes (Fig. 2b). At 6 hpt, several large patches of endocytosed polyplexes were found free of SDC1 clusters (Fig. 2a). The endocytosed polyplexes appeared to be concentrated in large particles as a result of the putative fusion of smaller ones. In stark contrast, only a few polyplexes were found endocytosed at 6 hpt in SDC2-expressing cells (Fig. 2b), and these were still associated with this

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**FIGURE 1. Polyplexes induce SDCs clustering.** HEK293 cells were transfected using 293fectin with GFP-tagged ΔCD4, SDC1, or SDC2-encoding plasmids. ΔCD4 was used as a non-HSPG control protein. After 24 hpt, polyplexes were added to cells expressing the indicated GFP-tagged protein. Redistribution of these proteins was followed by fluorescence microscopy. Formation of clusters could be easily monitored for SDC1 and SDC2 10 min after polyplex addition, whereas no clusters could be observed for ΔCD4. To explore the role of lipid rafts in polyplex-induced clustering, ΔCD4-, SDC1-, or SDC2-expressing cells were treated with 10 μM MβCD for 1 hpt. Polyplexes were then added to cells, and cluster formations were followed by fluorescence microscopy. Compared with polyplex addition in untreated cells, no clusters could be observed in MβCD-treated cells. Scale bar = 10 μm.

**FIGURE 2. Fate of SDC polyplex complexes.** SDC1-expressing (a) or SDC2-expressing (b) cells were plated for 1 h on MatTek 3.5-mm Petri dishes, RITC-labeled polyplexes were then added to cells. At the indicated time points, cells were fixed for 10 min by 4% formaldehyde. Nuclei (blue) were stained using the fluorescent dye Hoechst 33342. Confocal microscopy analysis was performed for each time point to determine the localization of GFP-tagged SDCs (green) and RITC-labeled polyplexes (PEI; red). Scale bar = 10 μm.
receptor. In both cases, polyplexes completely free of SDCs could be found in the cell cytoplasm at 24 hpt (Fig. 2, a and b). In agreement with a recent report showing that entry of polyplexes into the nucleus occurs during mitosis when the nuclear membrane is temporarily disrupted (13), some polyplexes were also found close to the chromatin in early post-mitotic cells (supplemental Fig. S3a), whereas some cells displayed polyplexes in their nuclei after mitosis (supplemental Fig. S3b).

**The Cytoplasmic Tail of SDC1 Is Not Required for Polyplex Endocytosis but Is Necessary for Gene Expression**—Because gene expression was not prevented but was slightly enhanced by overexpression of SDC1, we next wanted to evaluate the importance of its various domains with regard to PEI-mediated transfection. We therefore generated a series of C-terminally GFP-tagged truncated forms of SDC1 (Fig. 3) termed SDC1ΔC2 (without the C2 domain), SDC1ΔVC2 (without the variable and C2 domains), SDC1ΔCyto (without the cytoplasmic tail), and eSDC1 (the SDC1 ectodomain fused to the CD4 transmembrane domain), which were transfected in HEK293 cells. After 24 h, confocal microscopy analysis revealed that all constructs were localized at the cell membrane, indicating that these deletions did not affect plasma membrane targeting.3 Cells expressing each construct were then transfected with polyplexes containing the pTT-DsRed vector, and the percentage of cells expressing both fluorescent proteins was determined by flow cytometry 24 hpt. The results are shown in Fig. 3a and are expressed relative to full-length SDC1-transfected cells, taken as 100%. In SDC1ΔC2-expressing cells, the percentage of DsRed-expressing cells dramatically dropped to a level similar to that obtained with SDC2 (~20%), indicating that the C2 domain is crucial for PEI-mediated transfection. A similar drop in DsRed-expressing cells was observed for SDC1ΔVC2, SDC1ΔCyto, and eSDC1 (Fig. 3a).

Confocal microscopy analysis showed that, as observed previously, 10 min after adding polyplexes to wild-type SDC1-expressing cells, PEI rapidly induced formation of clusters with all truncated forms of SDC1 except for eSDC1.3 Interestingly, at 6 hpt (Fig. 3b), all constructs but eSDC1 were endocytosed and colocalized with polyplexes. In contrast to SDC2, these observations indicate that the inhibitory effect of these constructs on cell transfection could not be due to delayed polyplex endocytosis, but suggest that the intracellular targeting of polyplexes was affected. These results also demonstrate that the C2 domain of SDC1 is critical for its gene delivery capacity.
The SDC2 Ectodomain, but Not Its Cytoplasmic Domain, Inhibits Gene Transfer—We next wanted to determine the role of the SDC1 and SDC2 ectodomains during PEI-mediated gene delivery. To address this issue, we constructed two chimeras termed eSDC1tcSDC2 and eSDC2tcSDC1 by exchanging the ectodomains of SDC1 and SDC2. Cells expressing the chimeras were transfected with polyplexes containing the pTT-DsRed vector. At 24 hpt, the percentage of cells expressing both fluorescent proteins was determined by flow cytometry. Results are expressed relative to SDC1 expressed alone, taken as 100%. Data are means ± S.E. (error bars) of triplicates. TM, transmembrane; V, variable. b, to determine the potency of SDC1/SDC2 chimeras in polyplex endocytosis, HEK293 cells were transfected with eSDC1tcSDC2- or eSDC2tcSDC1-encoding plasmids using 293fectin. At 24 h post-293fectin transfection, SDC-expressing cells were transfected using BPEI-RITC complexed to the pTT5 vector. At 6 hpt, the localization of fluorescent proteins (green) and polyplexes (red) was determined by confocal microscopy. Scale bar = 10 μm.

FIGURE 4. Competence of the SDC1/SDC2 chimera in endocytosis and gene expression. a, to determine the impact of SDC1/SDC2 chimera expression on transfection efficiency, HEK293 cells were transfected with eSDC1tcSDC2- or eSDC2tcSDC1-encoding plasmids using 293fectin. At 24 h post-293fectin transfection, cells were transfected with polyplexes containing the pTT5-DsRed vector. At 24 hpt, the percentage of cells expressing both fluorescent proteins was determined by flow cytometry. Results are expressed relative to SDC1 expressed alone, taken as 100%. Data are means ± S.E. (error bars) of triplicates. TM, transmembrane; V, variable.

b, to determine the potency of SDC1/SDC2 chimeras in polyplex endocytosis, HEK293 cells were transfected with eSDC1tcSDC2- or eSDC2tcSDC1-encoding plasmids using 293fectin. At 24 h post-293fectin transfection, SDC-expressing cells were transfected using BPEI-RITC complexed to the pTT5 vector. At 6 hpt, the localization of fluorescent proteins (green) and polyplexes (red) was determined by confocal microscopy. Scale bar = 10 μm.

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Confocal microscopy analysis was performed with chimera-expressing cells following polyplex addition. As observed previously for SDC1 and SDC2, clusters of both chimeras colocalizing with RITC-labeled polyplexes could be observed 10 min following polyplex addition.3 Interestingly and in stark contrast to observations obtained wild-type SDC2, endocytosis of both chimera-polyplex complexes could be observed at 6 hpt (Fig. 4b). However, although endocytosed polyplexes were almost completely dissociated from eSDC1tcSDC2, as already observed for wild-type SDC1, they remained fully associated with eSDC2tcSDC1 (as for wild-type SDC2) (see Fig. 2b, 6 h panel). This suggests that the ectodomain of SDC2 is responsible for the negative effect on polyplex endocytosis and gene expression.

SDC2 Exerts a Dominant-negative Effect on Transfection—As both SDC1 and SDC2 have the capacity to bind polyplexes and form clusters but dramatically differ in terms of polyplex-induced endocytosis and transfection efficiency, we next examined the impact of their coexpression on these parameters. Cells coexpressing SDC1 and SDC2 were transfected with polyplexes containing the pTT-DsRed plasmid. At 24 hpt, the percentage of double-labeled cells (green for SDC expression and red for DsRed expression) was determined by flow cytometry. The results are expressed relative to the SDC1 control, set as 100%, where 50% of the coding plasmid was replaced with empty pTT5 vector to maintain the DNA/PEI ratio of 1/2. Coexpression of SDC2 with SDC1 resulted in a ~60% reduction in transfection efficiency compared with expression of SDC1 alone, indicating that SDC2 has a negative effect on SDC1-mediated PEI transfection (Fig. 5a).

As PEI-mediated transfection in SDC2-expressing cells was probably prevented as a direct or indirect result of a slowed polyplex endocytosis, confocal microscopy analysis was performed after addition of RITC-labeled polyplexes (Fig. 5b). To be able to distinguish between SDC1 and SDC2, GFP fused at the C-terminal end of the full-length SDC2 protein was replaced with BFP. At 10 min post-transfection, GFP-tagged SDC1, BFP-tagged SDC2, and RITC-labeled polyplexes all colocalized in clusters at the plasma membrane.3 Interestingly, SDC1-SDC2-polyplex complexes were hardly detected
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To achieve high levels of recombinant protein expression, the ideal non-viral gene delivery vehicle should possess the following properties: proficient binding to and penetration of target cells, capacity to bypass or escape the endocytotic pathways, efficient routing to the nucleus, and capacity to dissociate from membrane proteins (46), including SDC1 and SDC4 (40, 41). Using MECD, we have shown that lipid raft integrity is also required for polyplex-induced clustering of SDC1 and SDC2. Preliminary results indicate that PEI-mediated gene expression is inhibited in a dose-dependent manner by MβCD treatment.3

Surprisingly, in our system, the impact of SDC1 and SDC2 expression on PEI-mediated gene expression differs dramatically. Although by their nature it was expected that all SDCs would potentially be membrane receptors for polyplexes, we found that whereas SDC1 potentiated PEI-mediated gene expression, SDC2 significantly compromised it. Confocal microscopy analysis revealed that endocytosis of polyplexes bound to SDC1 occurred rapidly, whereas that of polyplexes bound to SDC2 took hours. Interestingly, many large SDC-free polyplexes could be observed in the cytoplasm at 24 hpt in both cases, suggesting that the presence of polyplexes in the cyto-

3 MβCD, a carbohydrate-specific lipid raft-disrupting agent, was used at concentrations of 10, 25, and 50 mM to inhibit PEI-mediated gene transfer in HEK293 cells.

FIGURE 5. Inhibition of SDC1 transfection by coexpression of SDC2. a, to determine the impact of SDC2 expression on SDC1 transfection efficiency, 293 cells were cotransfected with SDC1- and SDC2-encoding plasmids using 293fectin. At 24 h post-293fectin transfection, cells were transfected by polyplexes containing the pTT5-DsRed vector. At 24 hpt, the percentage of cells expressing both fluorescent proteins was determined by flow cytometry. Results are expressed relative to SDC1 expressed alone, where 50% of SDC1 plasmid was replaced with the pTT5 vector, and considered as 100%. Data are the means ± S.D. (error bars) of triplicates. b, to determine the impact of SDC2 expression on SDC1-polyplex endocytosis, 293S cells were cotransfected with SDC1-GFP- and SDC2-BFP-encoding plasmids using 293fectin. At 24 h post-293fectin transfection, SDC-expressing cells were transfected using BPEI-RITC complexed to the pTT5 vector. At 6 hpt, the localization of SDC1 (green), SDC2 (blue), and polyplexes (red) was determined by confocal microscopy. Scale bar = 10 μm.

within cells at 6 hpt, as they remained mainly at the cell surface, as observed previously when SDC2 was expressed alone. This result dramatically contrasts with the profile obtained when SDC1 was expressed alone (Fig. 2a), suggesting that SDC2 has a dominant-negative effect on SDC1-mediated polyplex endocytosis and gene transfer to the nucleus.

DISCUSSION

To achieve high levels of recombinant protein expression, the ideal non-viral gene delivery vehicle should possess the following properties: proficient binding to and penetration of target cells, capacity to bypass or escape the endocytotic pathways, efficient routing to the nucleus, and capacity to dissociate from plasmid DNA once in the nucleus. Compared with viruses, which have evolved to enable efficient infection, PEIs display a relatively weak gene transfer capability. The underlying mechanisms responsible for membrane binding and subsequent endocytosis of polyplexes remain largely unknown. In particular, the identity of membrane receptor(s) for polyplexes and the intracellular fate of receptor-polyplex complexes remain to be clearly elucidated.

It has been shown that HSPGs are involved in the cellular uptake of pollysine-nucleic acid and cationic lipid-nucleic acid complexes (42). In mammals, the dominating cell-surface HSPGs are syndecans and glypicans. These molecules are involved in many cellular processes such as differentiation, adhesion, and migration (19). Most of these processes are induced following HSPG binding to various extracellular proteins such as matrix proteins, proteases and their inhibitors, lipases, lipoproteins, and growth factors and their receptors. In the HSPG-negative (and difficult to transfect) cell line Raji, SDC1 overexpression has been found to promote cationic lipid-mediated transfection (43). However, no study to date has examined the role of SDCs in PEI-mediated gene delivery. In addition, whether all SDCs share the same capacity to bind and endocytose polyplexes in a way that ultimately leads to efficient gene expression has not been investigated. In this work, we have demonstrated that this is not the case, as SDC1 and SDC2 have opposite effects on PEI-mediated gene transfer in HEK293 cells. As HEK293 cells do not seem to express detectable levels of SDC1 (as observed following Western blot detection and reverse transcription-PCR), the natural receptor(s) to which polyplexes bind and enter cells is not yet known. Nonetheless, the levels of naturally occurring receptor(s) seem not to be limiting in HEK293 cells, as only a slight but significant improvement in gene transfer was observed following SDC1 overexpression.

Clustering of SDCs appears to be an important initial step that mediates their internalization. For example, Fuki et al. (44) reported that internalization of lipase-enriched lipoproteins by SDC1 requires the formation of such clusters. Using GFP-tagged SDC1 and SDC2, we have clearly shown that addition of polyplexes rapidly and efficiently induces formation of clusters that colocalize together. These results are consistent with the expected function of SDCs in PEI-mediated transfection and provide the first direct evidence demonstrating that PEI-DNA complexes bind to and efficiently induce clustering of SDCs. On the other hand, no cluster could be observed following 293fectin or Lipofectin addition, suggesting that cationic liposome-mediated transfection does not involve SDC clustering. These observations appear to contrast a previous study in which HSPGs and particularly SDC1 were shown to favor cationic liposome-DNA complex-based gene delivery (43). However, as no evidence of lipoplex-induced SDC1 clustering was provided, it is possible that HSPGs simply promote lipofection by enhancing recruitment of lipoplexes at the cell surface. In addition, it has been reported that the role of HSPGs in cationic lipid-mediated transfection is mostly to protect cells against lipid-mediated cytotoxicity (45).

The integrity of lipid rafts has been shown to be essential for the clustering and biological activity of a wide variety of membrane proteins (46), including SDC1 and SDC4 (40, 41). Using MβCD, we have shown that lipid raft integrity is also required for polyplex-induced clustering of SDC1 and SDC2. Preliminary results indicate that PEI-mediated gene expression is inhibited in a dose-dependent manner by MβCD treatment.3
plasm is not sufficient to enable transfection of cells. This is further supported by previous observation showing that plasmid DNA encoding BFP remains in the cytosol for up to 96 hpt in both BFP-expressing and BFP-nonexpressing cells (47). As SDC1 was tagged with GFP at its C-terminal end, we cannot rule out the possibility that polyplexes were still associated with cleaved SDC1 ectodomains at 24 hpt, although the inhibitory effect of SDC2 was not rescued by SDC1 coexpression. This shows that SDC2 expression has a dominant-negative effect on both overexpressed SDC1 and the naturally expressed receptor(s) present in HEK293 cells. Confocal microscopy analysis also revealed that SDC1 and SDC2 colocalized during PEI-mediated transfection, suggesting that their hetero-oligomerization may occur. The kinetics of polyplex endocytosis in SDC2/SDC1-expressing cells is, as for SDC2, greatly delayed compared with SDC1-expressing cells. This suggests that, in addition to endocytosis, the kinetics by which polyplexes enter cells may be an important parameter for efficient gene transfer. Taken together, these results support the possibility that the delay in polyplex endocytosis could be responsible for the weak transgene expression observed in SDC2-expressing cells, but further investigation is required to confirm this hypothesis.

The fact that SDC1 but not SDC2 is able to trigger transgene expression raises the question regarding SDC domains that are important for polyplex-mediated clustering, endocytosis, and gene expression. Using a series of SDC1 deletion mutants, we have shown that the C2 domain is crucial for PEI-mediated gene expression, but not for clustering and endocytosis. As the C2 domain can bind proteins containing a PDZ domain such as syntenin (27), CASK/Lin-2 (28), synbindin (29), and synectin (26), syndecan-binding PDZ proteins may play a critical role in PEI-mediated transfection. Although binding to C-terminal peptides of partner proteins appears to be the typical mode of PDZ domain interaction, these domains can also interact with internal peptide sequences (48). It is thus possible that the presence of GFP at the C termini of SDC1 and SDC2 does not interfere with their interaction with PDZ proteins such as syntenin. In fact, the presence of the SDC1 C2 domain is critical because its deletion significantly impairs PEI-mediated gene expression, but not clustering or endocytosis. This is in agreement with a previous study showing that abrogation of the SDC1 cytoplasmic domain is required for PEI-mediated transfection and also provides a new function for SDC1 and SDC2. Our results also suggest that the role of each member of the HSPG family in PEI-mediated gene delivery should be investigated individually rather than collectively. In addition, this work implies that the nature of the SDCs expressed in a particular tissue or cell line would have a significant impact in gene therapy applications.

In conclusion, we have shown here for the first time that SDC1 and SDC2 are directly involved in polyplex binding and that SDC2 strongly delays polyplex endocytosis and inhibits PEI-mediated gene expression. Taken together, this work sheds some light on the mechanisms involved during polyplex-mediated transfection and also provides a new function for SDC1 and SDC2. Our results also suggest that the role of each member of the HSPG family in PEI-mediated gene delivery should be investigated individually rather than collectively. In addition, this work implies that the nature of the SDCs expressed in a particular tissue or cell line would have a significant impact in gene therapy applications.

Acknowledgments—We are grateful to Drs. Alan C. Rapraeger (University of Wisconsin-Madison) and Joachim Schulz (University of Leuven) for providing full-length cDNAs of SDC1 and SDC2 constructs, respectively; Dr. Guido David for providing anti-syndecan-4 antibody (F94-8G3); and Dr. Mini Thomas (Massachusetts Institute of Technology) for providing the cross-linked PEIs. We are especially indebted to Christian Charbonneau for expert assistance with confocal microscopy and to Lucie Bourget for help with flow cytometry analyses. We are grateful to Dr. Mark Abramovitz (VM Institute of Research, Montreal, Canada) for critical reading of the manuscript and Drs. Joseph D. Schrag (Biotechnology Research Institute) and Pascale Zimmermann (University of Leuven) for helpful comments.

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