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Summary

Full activity of fibroblast growth factors (FGFs) requires their internalization in addition to the interaction with cell surface receptors. Recent studies have suggested that the transmembrane proteoglycan syndecan-4 functions as a FGF2 receptor. In this study we investigated the molecular basis of syndecan endocytosis and its role in FGF2 internalization in endothelial cells. We found that syndecan-4 uptake, induced either by treatment with FGF2 or by antibody clustering, requires the integrity of plasma membrane lipid rafts for its initiation, occurs in a non-clathrin-, non-dynamin-dependent manner and involves Rac1, which is activated by syndecan-4 clustering. FGF2 was internalized in a complex with syndecan-4 in 70 kDa dextran-containing endocytic vesicles. FGF2 and syndecan-4 but not dextran endocytosis were blocked by the dominant negative Rac1 while amiloride and the dominant-negative Cdc42 blocked internalization of dextran in addition to FGF2 and syndecan-4. Taken together, these results demonstrate that FGF2 endocytosis requires syndecan-4 clustering-dependent activation of Rac1 and the intact CDC42-dependent macropinocytic pathway.

Key words: Rac1, Macropinocytosis, Heparan sulfate, Growth factors, Signaling

Introduction

Fibroblast growth factors (FGFs) play a multitude of roles in modulation of cell growth, migration and differentiation (Ornitz and Itoh, 2001). The signaling activity of FGFs involves interaction with high affinity tyrosine kinase receptors and cell surface heparan sulfates. The latter molecules have long been thought to play a role as co-receptors for heparin binding growth factors such as FGFs. In particular, studies of FGF2 signaling established its dependence on the presence of cell surface heparan sulfates (Rapraeger et al., 1994) that are carried on membrane-bound core proteins, syndecans and glypicans (Rosenberg et al., 1997; Zimmermann and David, 1999). However, more recent studies have suggested that some core proteins themselves, and not just their heparan sulfate chains, may play an important role in signal transduction. Of these, syndecan-4 has emerged as a signaling receptor because of its ability to bind and activate protein kinase C α (PKCα) upon FGF2-induced oligomerization (Simons and Horowitz, 2001). This association of syndecan-4 with PKCα puts the proteoglycan in a category similar to other transmembrane receptors, such as integrins, that do not possess intrinsic catalytic activity, but are associated with kinases or phosphatases.

A substantial body of literature suggests that full activity of FGFs requires not only receptor interactions but also their internalization (Goldfarb, 2001) and that the latter may proceed via heparan sulfate proteoglycans-dependent and -independent pathways (Citores et al., 1999; Gleizes et al., 1995; Grieb and Burgess, 2000; Roghani and Moscatelli, 1992). Internalization of trans-membrane proteins plays an important role in multiple cellular processes. In particular, syndecan endocytosis has been suggested as an important regulator of lipoprotein uptake (Fuki et al., 1997; Williams, 2001). At the same time, the details of FGF endocytosis and the involvement of specific proteoglycans have not been established.

Recent studies demonstrating direct syndecan-4 involvement in FGF2 signaling (Horowitz et al., 2002; Volk et al., 1999) raise the possibility that this proteoglycan may also play a role in its internalization thereby providing an additional avenue for regulation of its activity. This possibility is bolstered by observations that syndecan-4 binding to soluble (e.g. growth factors) or extracellular matrix ligand or cell exposure to mechanical stress results in its endocytosis (Li and Chaikof, 2002; Tkachenko and Simons, 2002). However, the link between syndecan-4 endocytosis and FGF2 internalization as well as molecular mechanisms of this process have not been defined.

To gain further insight into syndecan-4 function, we studied its clustering-induced endocytosis in endothelial cells. We find that following oligomerization, the core protein is internalized from the plasma membrane in a lipid raft-dependent, clathrin- and dynamin-independent manner by a process that is amiloride sensitive and requires activation of the small GTPase Rac1. Furthermore, syndecan-4 clustering induced by FGF2 resulted in FGF2 internalization together with syndecan-4 and 70 kDa dextran in the same endocytic vesicles. FGF2 and syndecan-4

but not dextran internalization was blocked by dominant negative Rac1, while amiloride and dominant-negative Cdc42 blocked internalization of not only FGF2 and syndecan-4 but also of 70 kDa dextran. This set of conditions shows that FGF2-induced syndecan-4 clustering leads to internalization of both proteins that proceeds via a macropinocytic pathway. Activation of Rac1 is required for the initiation of syndecan-4 endocytosis that also requires a fully functional macropinocytic pathway.

Materials and Methods

Antibodies and reagents

Human non-immune IgG and F(ab′)$_2$ fragment-specific goat anti-human Igb F(ab′)$_2$ antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Alexa-488 and Alexa-647 labeling kits, anti-HA epitope antibodies directly labeled by Alexa-488 and Zenon Alexa Fluor-488 mouse IgG$_1$ labeling kit, BODIPY® FL C5-ganglioside GM1 complexed to bovine serum albumin (BSA) and dextran-rhodamineB 70 MW were purchased from Molecular Probes. Alexa-488-conjugated inactive variant of the protein proaerolysin (FLAER) was purchased from Protox Biotech (Victoria, British Columbia, Canada). Mouse anti-c-myc epitope-tagged monoclonal antibody (mAb) was purchased from Sigma. Chicken IgY anti-extracellular domain of syndecan-4 was fluorescently labeled with Alexa-488 according to the manufacturer's protocol (Molecular Probes). Human recombinant FGF2 was courtesy of Chiron Corporation (Sunnyvale, CA, USA). All other reagents were purchased from Sigma.

cDNA constructs and transfections

Fc receptor-syndecan-4 chimera (FcR-S4) construct was previously described (Tkachenko and Simons, 2002), eGFP-Cdc42-N17 (dominant negative), eGFP-Cdc42-I61(constitutively active), eGFP-Rac1b-N17 (dominant negative), eGFP-Rac1b-I61(constitutively positive) and GPI-GFP plasmids were provided by M. Way (Cancer Research UK) and S. Mayor (Sabharanjak et al., 2002) (National Centre for Biological Sciences, Bangalore, India). RhoA T19N (dominant negative) and RhoA G14V (constitutively active) were purchased from Guthrie Research Institute and were recloned into pIRES2-eGFP bicistronic vector (Clontech, CA, USA). eGFP-clathrin light chain construct was a kind gift from J. Keen (Thomas Jefferson University, Philadelphia, PA, USA), AP180C was from H. McMahon, dynamin1^{K44A} from S. Schmid (Scripps Research Institute, La Jolla, CA, USA) and dynamin2^{k44a} construct was from M. A. McNiven (Cao et al., 1998). Rat fat pad endothelial cells (RFPEC) were cultured in DMEM (Invitrogen, CA, USA) as described previously (Horowitz et al., 2002).

Endocytosis assays

To label vesicles that undergo macropinocytosis, rat fat pad endothelial cells (RFPEC) were incubated on ice in 1% BSA-DMEM for 30 minutes. Neutral 70-kDa Dextran (rDx)-Rhodamine B was added to a final concentration of 0.5 mg/ml and incubated with cells at 37°C in 5% CO_2 for 20 minutes. To label clathrin-coated endocytic vesicles cells were incubated in 1% BSA-DMEM and then loaded with 1 μg/ml of DiI-LDL or 25 μg/ml of transferrin-Alexa647 (TfR) followed by incubation for 30 minutes on ice, washed once and then incubated at 37°C for 20 minutes in 5% CO_2. Labeling of endogenous GPI-anchored proteins by FLAER was done as previously described (Tkachenko and Simons, 2002).

FGF2 labeling with Alexa Fluor 647

In order to protect the syndecan-4 binding sites, FGF2 (1 mg) was adsorbed to 0.5 ml bed-volume heparin-Sepharose beads (Amersham Biosciences). The beads were washed with ice-cold 0.1 M $NaHCO_3$, re-suspended in 1 ml of ice-cold 0.1 M $NaHCO_3$, pH 8.2. AlexaFluor647 succinimidyl ester was added (0.5 mg in 0.2 ml 0.1 M $NaHCO_3$) and the mixture was shaken for 1 hour at 4°C. The supernatant was drained and the column washed with 4 ml TBS and 2 ml 10 mM Tris/0.3 M NaCl pH 7.5. Labeled FGF2 was eluted with 2 ml 10 mM Tris/2 M NaCl pH 7.5 and desalted using two Hi-Trap desalting cartridges connected in series and elution with Tris-buffered saline. The FGF2-containing fraction was concentrated in an Amicon 10-kDa centrifugal filter (Millipore) and protein concentration was determined by spectrophotometry. The labeling ratio dye:FGF2 was 0.5:1, which was intended to be low as not to interfere with the biological function of FGF2.

Syndecan clustering

Antibody and FGF2 clustering were performed as described previously (Tkachenko and Simons, 2002). Briefly, RFPEC stably expressing FcR-syndecan-4 were plated and grown overnight in 10% FBS-DMEM. For antibody clustering, cells were washed with DMEM and incubated in the presence of biotinylated non-immune IgG (1 μg/ml) for 15 minutes in DMEM supplemented with 1% BSA at 37°C. Then cells were washed with ice-cold DMEM and incubated for another 30 minutes on ice with anti-human F(ab′)$_2$-Cy3 (3 μg/ml) in DMEM-1% BSA followed by wash as above and incubation at 37°C with 5% CO_2 in DMEM 1% BSA for various times. To distinguish plasma membrane syndecan-4 from internalized syndecan-4, the cells were then washed with ice cold medium and incubated with steptavidin-Cy5 or streptavidin-Alexa488 (1:400 dilution) for 30 minutes at 4°C. At this temperature, streptavidin has access only to the surface bound biotinylated IgG, or the IgG that is in vesicles open to the cell surface. For FGF2 labeling, cells were washed with ice-cold DMEM twice and incubated for 30 minutes on ice in DMEM in the presence of 50 ng/ml of FGF2-Alexa Fluor. After washing cells were incubated in DMEM-1% BSA for various times. To remove remaining cell surface FGF2, cells were place on ice and incubated with 10 mg/ml of heparin in DMEM.

Immunofluorescence microscopy

RFPEC were plated in chamber slides (Nalge Nunc), fixed with 2% paraformaldehyde for 10 minutes at room temperature, then washed twice with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100/PBS for 10 minutes, washed again as above, blocked with 3% BSA/PBS for 30 minutes, and incubated with primary antibodies (1 μg/ml) in 1% BSA/PBS overnight at 4°C. The slides were then washed four times as above, and incubated for 1 hour with 10 μg/ml of the appropriate reporter-conjugated secondary antibodies, washed again as before, and mounted with ProLong medium (Molecular Probes). Fluorescence microscopy was carried out on live cells as described previously (Tkachenko and Simons, 2002). Imaging was carried out using Bio-Rad MRC-1024 and Ziess LSM 510 Meta laser confocal system microscopes with Zeiss 63× objectives. Image analysis was done using Adobe Photoshop 7.0 (Adobe Systems Inc.), AutoDeblur 9.1 (Auto Quant Imaging Inc.) and Image J software (NIH).

Toxin treatment and plasma membrane cholesterol depletion

RFPEC were incubated with 20 ng/ml of *Clostridium difficile* toxin B for 3 hours or 100 ng/ml exotransferase C3 for 12-16 hours in the normal growth medium. 5-(*N*-ethyl-*N*-isopropyl) amiloride treatment of RFPEC was performed as described previously (Muro et al., 2003). Plasma membrane cholesterol depletion was accomplished by pretreatment of cultured cells with 10 mM methyl β-cyclodextrin (MβCD), for 30 minutes at 37°C followed by a wash with DMEM and standard clustering procedure. MβCD removes unesterefied cholesterol from the cell membrane (Rodrigueza et al., 1997).

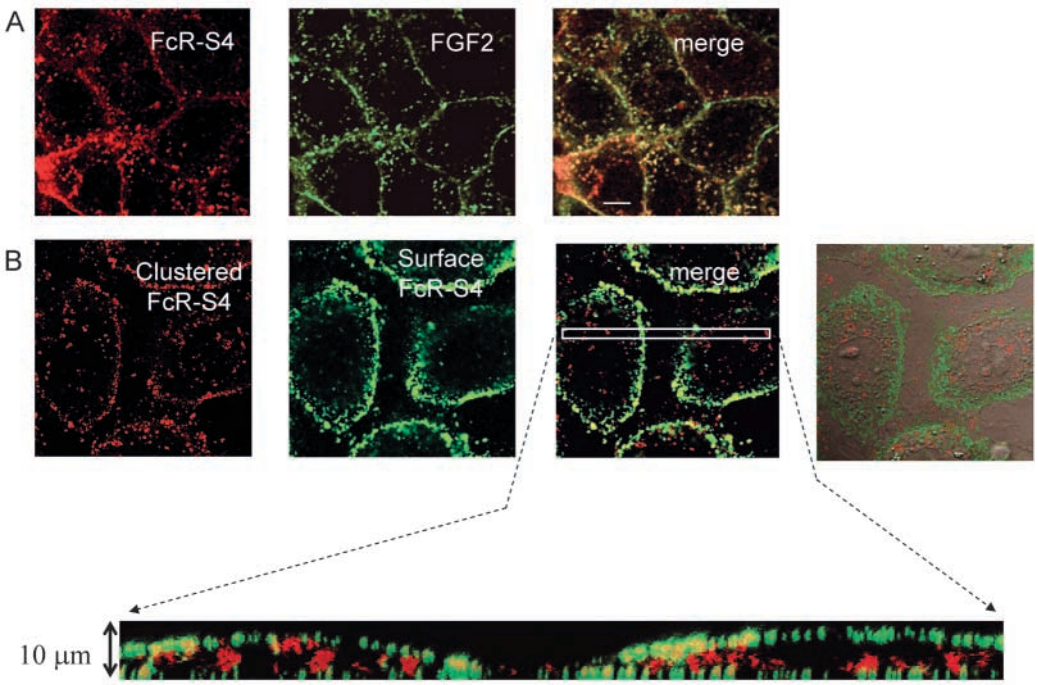


Fig. 1. Internalization of the native and chimera syndecan-4 proteins. (A) RFPEC cells expressing the FcR-S4 construct were decorated with Cy3-labeled (red) human non-immune IgG (niIgG) and then clustered with AlexaFluor 647-labeled (green) FGF2. IgG decorated FcR-S4 chimeras are present on the surface of the cells and in the cytoplasm 20 minutes after FGF2 clustering (red). FGF2 is present both on the cell surface and in the cytoplasm. Note co-localization (yellow) of internalized FGF2 and FcR-S4 chimeras (merged image). This observation is consistent with FGF2-induced oligomerization of FcR-S4 native S4 heterodimers. (B) FcR-S4-expressing cells decorated with biotinylated niIgG followed by clustering with Cy3-labeled clustering antibodies (red). Biotinylated niIgG remaining on the cell surface was visualized by streptavidin-Cy5 (green). Dual stain on the merged image shows cell surface localized FcR-S4 whereas single red stain shows internalized FcR-S4. Right figure shows the merged color image overlaid with a DIC image. Lower figure shows Z-plane projection of the area in the white rectangle.

Quantitative measurements of endocytic uptake

Endocytic uptake of dextran, transferrin, GPI-GFP, GM1-BODIPY, FcR-syndecan 4 and FGF2 was quantified as previously described (Sabharanjak et al., 2002). In brief, 10-15 images were acquired from two wells of the same experiment and the number of cells showing high level of uptake relative to untransfected cells was scored. The data are presented as mean of three or four independent experiments, each with 20 or more cells.

In addition, the effect of various toxin treatments on syndecan-4 and transferrin uptake was measured by flow cytometry. For these experiments (n=3) cells were grown to ~70-80% confluence on 24-well plates. Toxin treatments, FcR-syndecan 4 and transferrin uptake assays were performed as described above. At the end of the experiment plates were placed on ice, washed once with ice-cold PBS pH 7.4 and then twice with ice-cold acidic PBS pH 2.5 for 30 seconds to remove cell-surface bound fluorescent ligands (Fuki et al., 2000). Cells were then trypsinized, washed once with PBS pH 7.4 and resuspended in DPBS pH 7.4 with 1% BSA. Flow cytometry was performed and analyzed as previously described (Tkachenko and Simons, 2002).

Rho GTPase pulldown assays

To determine activation of Rho, Rac and Cdc42 after FcR-S4 clustering, EZ-detect Rho and Rac1 activation kits (Pierce) were used. In brief, cells were seeded on fibronectin-coated 100 mm dishes and grown to 80% confluency then starved for 24 hours in DMED 0.5% FBS. Following antibody clustering performed as described above, cells were lysed in 250 μl lysis/binding/washing buffer including proteinase inhibitors (Pierce). As positive and negative controls, four clarified non-clustered cell lysates (500 μg) were treated with either 0.1 mM GTPγS or 1.0 mM GDP in the presence of 10 mM EDTA, pH 8.0 at 30°C for either 15 minutes (to activate or inactivate Rac1 and Cdc42) or 30 minutes (to activate or inactivate Rho). The nucleotide exchange reaction was terminated by adding $MgCl_2$ and placing samples on ice.

The cell lysates (500 μg) were then incubated with either GST-Pak1-PBD (to pull down active Cdc42 or Rac1) or GST-Rhotekin-RBD (to pull down active Rho) in the presence of SwellGel-immobilized glutathione at 4°C for 1 hour in a spin column. After incubation, the mixture was centrifuged at 8,000 ***g*** to remove the unbound proteins. The resins were washed three times with lysis/binding/wash buffer and the sample was eluted by adding 50 μl of 2× SDS sample buffer and boiling at 95°C for 5 minutes. Half (25 μl) of the sample volumes were analyzed by SDS-PAGE and transferred to a PDVF membrane. The active Rac1 and Rho were detected by western blotting using a specific mouse monoclonal antibody. A goat anti-mouse antibody conjugated with HRP was used as the secondary antibody. For the detection of Cdc42, anti-Cdc42 monoclonal antibody (Upstate) was labeled using the Zenon Alexa-488 mouse IgG1 labeling kit and applied to the rac1/cdc42 PVDF membrane. Detection was performed using West Pico Chemiluminescent Substrate (Rho and Rac1) (Pierce) or fluorometry (Cdc42) followed by exposure to X-ray film or detection of fluorescence in a Typhoon 9410 (Amersham Biosciences) detector, respectively.

Results

Clustering initiates syndecan-4 endocytosis

To study plasma membrane syndecan-4 internalization, we used live confocal laser microscopy to track the core protein in endothelial cells before and after FGF2 and/or antibody-induced clustering. To help visualize the fate of the native syndecan-4, we took advantage of heterodimerization between the wild-type syndecan-4 and the FcR-S4 chimeras previously demonstrated in the FcR-S4-expressing RFPEC (Tkachenko and Simons, 2002). In quiescent endothelial cells both the FcR-S4 chimera and the wild-type syndecan-4 were similarly expressed on the plasma membrane (Fig. 1A). FcR-S4-expressing RFPEC were treated with Cy3-labeled IgG. This decorates chimeras expressed on the cell plasma membranes

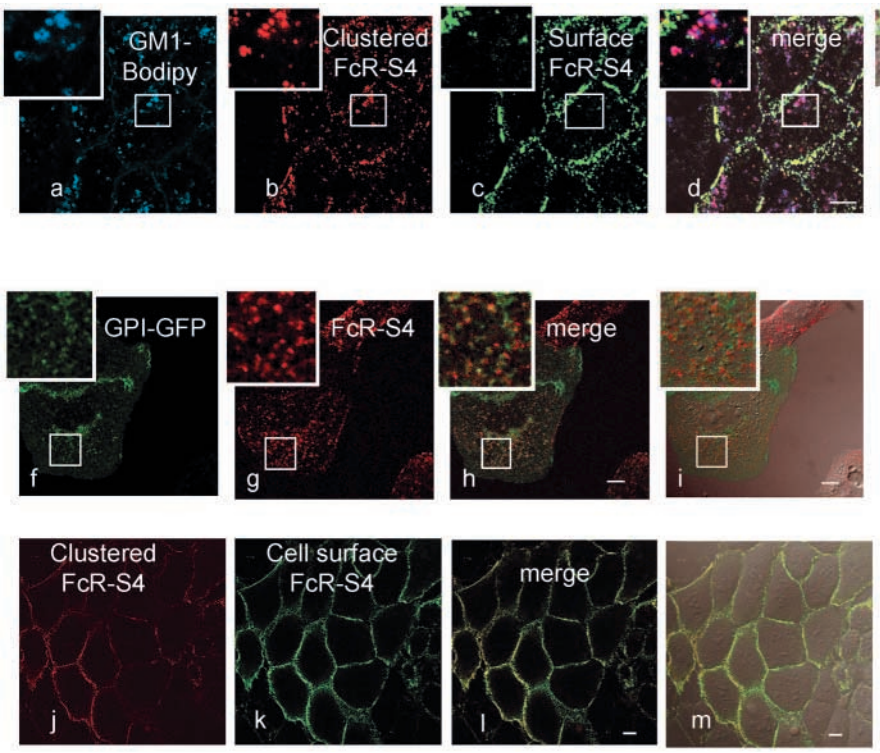


Fig. 2. Syndecan-4 is internalized from lipid raft regions of the plasma membrane. Confocal microscopy of syndecan-4 endocytosis was carried out in RFPEC expressing the FcR-S4 chimera. The raft portion of the plasma membrane was labeled with BODIPY GM1 (blue) (a) or by transient expression of GPI-GFP construct (f). FcR-S4 chimeras were decorated with biotinylated niIgG and clustering was then initiated by the addition of Cy3-F(ab′)$_2$ fragments (red). Five minutes after the initiation of antibody clustering, all internalized FcR-S4 chimeras (b) were seen in close association with GM1-BODIPY (magenta, a,d,c) membrane domains or with GPI-GFP (yellow, h and i). (e,i) Merged color images (d and h, respectively) overlaid with DIC images. The depletion of membrane cholesterol with MβCD fully blocks clustering-induced FcR-S4 internalization (j,k,l,m). Scale bars, 10 μm.

but does not initiate their internalization (not shown). The cells were then treated with FGF2. Twenty minutes after FGF2 exposure, there was a substantial internalization of FcR-S4 chimeras (Fig. 1A, left panel) and of FGF2 itself (Fig. 1A, middle panel). The merged image demonstrates significant intracellular co-localization of FGF2 and FcR-S4 signals (Fig. 1A, right panel). This suggests that FcR-S4 chimeras that have formed heterodimers with the native syndecan-4 core protein, were internalized when the latter was oligomerized by the FGF2 treatment, as we have shown previously (Tkachenko and Simons, 2002).

Similarly, F(ab′)$_2$-mediated clustering of IgG-decorated FcR-S4 chimeras also induced their internalization (Fig. 1B). Overall, kinetics of FcR-S4 chimera endocytosis was similar to that of the endogenous syndecan-4 with 50% of both proteins internalized within 20 minutes of antibody-mediated clustering.

Syndecan-4 endocytosis depends on the integrity of the lipid rafts and occurs in a clathrin and dynamin-independent manner

We have previously shown that FGF2-induced syndecan-4 or antibody-induced FcR-S4 chimera oligomerization result in redistribution of approximately 50% of the total plasma membrane content of these proteins to the lipid rafts (Tkachenko and Simons, 2002). To determine whether syndecan-4 endocytosis proceeds from the rafts or a non-raft portion of the plasma membrane, we used confocal microscopy of

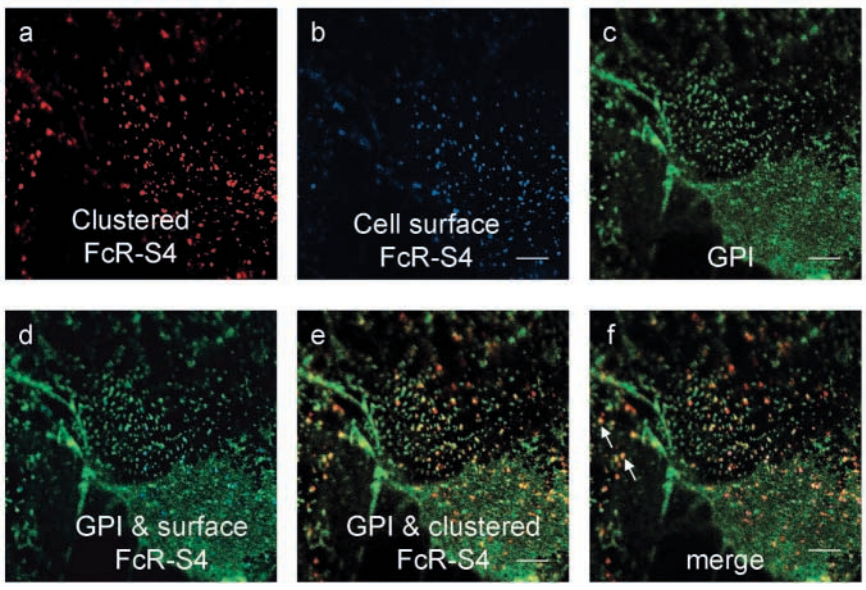


Fig. 3. Syndecan-4 is internalized with endogenous GPI-anchored proteins. (a) FcR-S4 expressing RFPES were labeled with FLAER, decorated by biotinylated niIgG and clustered by Cy-3-F(ab′)$_2$. (b) Five minutes after clustering, cells were placed on ice and the cell surface FcR-S4 was detected with streptavidin-Cy5. (c) FLAER-labeled GPI-anchored proteins. (d-f) Cell surface (d,f) and internalized (e,f) FcR-S4 demonstrate high level of colocalization with GPI-anchored proteins. Arrows in f point to examples of FcR-S4- and FLAER-labeled internalized vesicles. Scale bars, 10 μm.

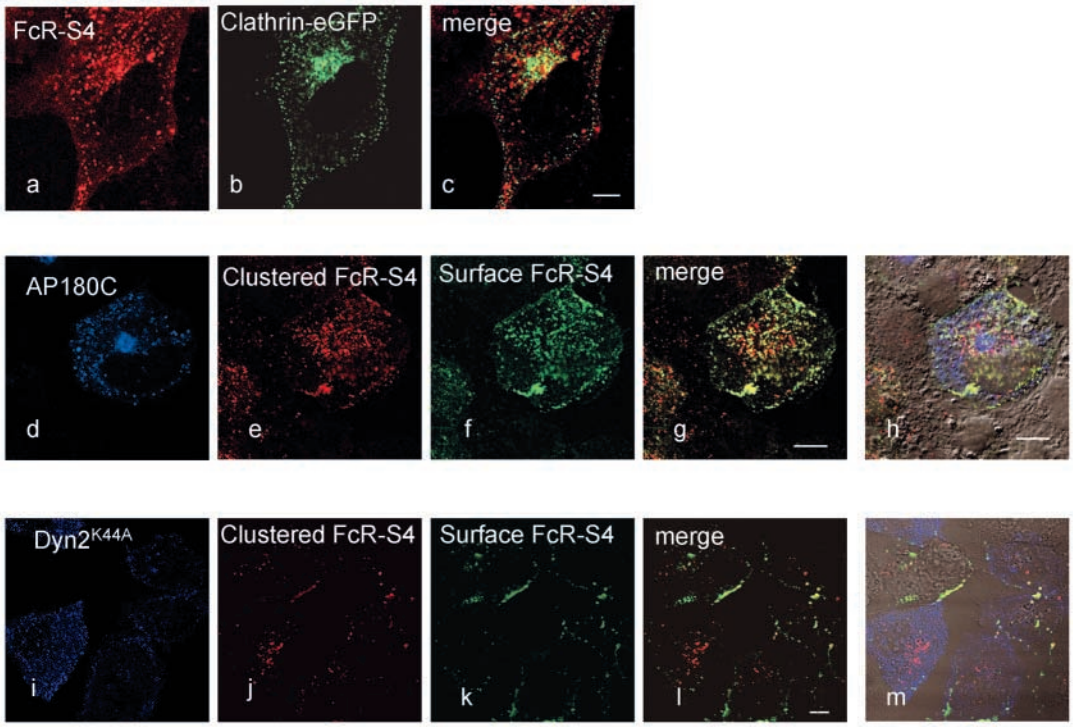


Fig. 4. Syndecan-4 is internalized in the clathrin- and dynamin-independent manner. (a-c) Syndecan-4 endocytosis was studied in FcR-S4 expressing RFPEC transiently transfected with various constructs. Antibody clustering of FcR-S4 chimeras carried out as described in Fig. 1 and 2 leads to internalization of syndecan-4 (a). Note the absence of co-localization of internalized syndecan-4 with clathrin in cells expressing clathrin-eGFP 5 minutes after clustering (b,c). The effect of clathrin dominant negative was studied in cells transiently expressing a c-myc tagged AP180C construct (d). Cell expressing AP180C demonstrates the same FcR-S4 internalization as non-transfected cells (e,f,g,h). The FcR-S4 remaining on the cell surface is shown in green and internalized FcR-S4 is in red. (i-m) The role of dynamin was studied in cells transiently expressing a dominant-negative HA-tagged dyn2^{K44A} construct (i). Note that the cell expressing Dyn2^{K44A} demonstrates high level of FcR-S4 internalization (r,i,l,m). h and m show the color images in g and l, merged with their respective DIC images. In g, h and i internalized FcR-S4 is in red, and in f,g,h,k,i FcR-S4 constructs remaining on the cell surface are in yellow and green. Scale bars, 10 μm.

live cells to track internalized FcR-S4 chimeras as well as BODIPY-GM1, a lipid raft marker.

Within 5 minutes after clustering with F(ab′)$_2$ fragments, most if not all internalized FcR-S4 chimeras co-localized with BODIPY-GM1 (Fig. 2a-e). To confirm this finding, we examined syndecan-4 endocytosis in RFPEC-expressing GPI-anchored GFP. Following antibody clustering of the FcR-S4 chimera, confocal microscopy of live cells demonstrated considerable co-localization between the internalized syndecan-4 chimeras and GPI-GFP (Fig. 2f-i). To confirm co-localization of internalized syndecan-4 with rafts proteins, we used FLAER to detect GPI-anchored proteins (Brodsky et al., 2000; Tkachenko and Simons, 2002). As in the GPI-GFP studies, we observed a high level of colocalization of internalized syndecan-4 with FLAER-labeled endogenous GPI-anchored proteins (Fig. 3). These results suggest, therefore, that essentially all internalized syndecan-4 originated from a region rich in lipid rafts. To confirm this conclusion, we used methyl β-cyclodextrin (MβCD) to deplete plasma membrane cholesterol content thereby preventing formation of lipid rafts. As expected, MβCD treatment completely prevented antibody-induced internalization of FcR-S4 chimeras (Fig. 2j-m).

Internalization of plasma membrane receptors can occur via clathrin-dependent or independent pathways. To determine the role played by clathrin in syndecan-4 internalization, we examined co-localization of the endocytosed syndecan-4 and clathrin-LC-eGFP and studied the effect of expression of the AP180C construct that has been shown to selectively block clathrin-dependent endocytosis (Ford et al., 2001) on syndecan-4 uptake. Confocal microscopy demonstrated no co-localization between syndecan-4 and clathrin (Fig. 4a-c). After transient transfection of AP180C into RFPEC, cells demonstrating AP180C expression showed, as expected, inhibition of clathrin-dependent transferrin uptake (a). Syndecan-4 endocytosis, however, was not affected by AP180C expression (Fig. 4d-h, Fig. 5a). These results suggest that syndecan-4 endocytosis occurs via a clathrin-independent pathway.

To further define the endosomal pathway involved in syndecan-4 trafficking, dynamin dominant-negative constructs specific for both dynamins, dyn1^{K44A} and dyn2^{K44A} were transiently expressed in RFPEC. While expression of either dynamin construct had little effect on syndecan-4 endocytosis (Fig. 4i-l, Fig. 5a), both dynamin dominant negatives inhibited clathrin-dependent transferrin uptake (Fig. 5a).

Syndecan-4 endocytosis requires Rac1

Rho GTPases are involved in a number of endocytic processes.

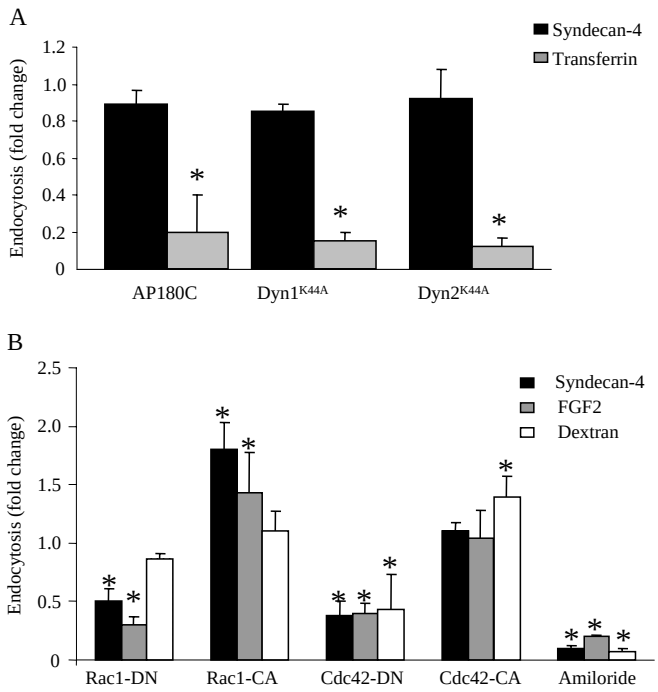


Fig. 5. Quantitative analysis of syndecan-4 endocytosis was carried out using confocal microscopy as described in the Materials and Methods. The results are presented as a fold change from baseline for each substance. (A) The effects of dominant negative constructs (AP180c) and dynamins 1 and 2 ($Dyn1^{K44A}$ and $Dyn2^{K44A}$) on clathrin-dependent uptake of syndecan-4 (black bars) and transferrin (gray bars). Note inhibition of transferrin but not syndecan-4 uptake. * $P<0.05$ versus baseline. (B) The effects of Rac1 and Cdc42 dominant negative and constitutively active constructs and amiloride treatment on FcR-syndecan-4 (black bars), FGF2 (gray bars) and dextran (white bars) uptake was examined in RFPEC. Note a significant inhibition of syndecan-4 and FGF2 uptake in Rac1-DN-expressing cells and increased uptake in Rac1-CA expressing cells. Cdc42-DN and amiloride inhibited uptake of all three substances. *$P<0.05$ versus baseline endocytosis.

To examine the involvement of these GTPases in oligomerization-induced syndecan-4 uptake, we first studied the effect of syndecan oligomerization on activation of different Rho family members. To this end, we examined the time course of activation of RhoA, Cdc42 and Rac1 in RFPEC expressing the FcR-S4 construct following antibody-induced chimera oligomerization. While there was no change in RhoA or Cdc42 activity, within 5 minutes there was a significant activation of Rac1 that gradually declined to baseline levels over the next 60 minutes (Fig. 6).

Since Rac1 activation preceded the activation of syndecan-4 endocytosis and reverted to baseline levels when its endocytosis was complete, we next examined whether Rac1 activation is indeed required for this process. Treatment of cells with *C. difficile* toxin B, inhibitor of all Rho GTPases, completely abolished all FcR-S4 endocytosis (Fig. 7a). At the same time, treatment with a RhoA inhibitor, C3 exotransferase, had no effect on this process (Fig. 7b). To further investigate which Rho family member is involved in syndecan endocytosis, dominant negative Rac1 and RhoA as well as constitutively active mutants of Rac1, Cdc42 and

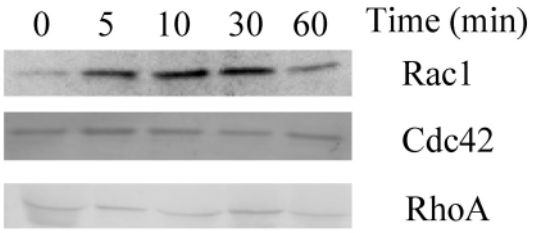


Fig. 6. Rac1 activation by syndecan-4 clustering. Time course of Rac1, RhoA and Cdc42 activation following antibody oligomerization of FcR-S4 chimeras. GST pull down assays with specific substrates were performed as described in the Materials and Methods. Note activation of Rac1 5 minutes after initiation of syndecan-4 oligomerization while neither RhoA nor Cdc42 activity is changed.

RhoA were transiently expressed in RFPEC expressing FcR-S4 chimeras and their effect on clustering-induced syndecan-4 endocytosis was then examined. Transient expression of a Rac1DN construct completely blocked FcR-S4 endocytosis while the expression of a Rac1 constitutively active construct markedly accelerated it (Fig. 8a, Fig. 5b). Transient expression of a Cdc42 dominant also downregulated syndecan-4 endocytosis while a Cdc42 constitutively active construct had no effect (Fig. 8b, Fig. 5b). At the same time, expression of RhoA dominant negative or constitutively active constructs had no effect on FcR-S4 internalization (Fig. 8c, Fig. 5b).

Syndecan-4 and FGF2 internalization proceeds via macropinocytosis

The involvement of Rac1 in syndecan-4 endocytosis suggests that the latter might proceed via a macropinocytic pathway. To evaluate this possibility FcR-S4-expressing RFPEC were treated with fluorescently labeled 70 kDa dextran and then syndecan clustering was induced by an antibody or by addition of fluorescently labeled FGF2 (Fig. 9a-c). Confocal microscopy of live cells demonstrated partial co-localization of internalized dextran and FcR-S4 (Fig. 9d) following antibody clustering, a finding consistent with the presence of FcR-S4 chimera in macropinocytic vesicles. Similarly, following FGF2 treatment of endothelial cells exposed to dextran, there was considerable co-localization of dextran and FGF2 signals (Fig. 9e) and both FGF2 and dextran were observed in syndecan-4-containing vesicles (Fig. 9f).

Since macropinocytosis is sensitive to amiloride, we examined the effect of this inhibitor on both syndecan-4 and FGF2 endocytosis. Pretreatment of cells with amiloride completely inhibited internalization of dextran, as expected (Fig. 5b). At the same time, it also inhibited both the antibody clustering-induced FcR-S4 chimera endocytosis (Fig. 9g-j) and FGF2 endocytosis (not shown, see Fig. 5b for quantification). However, amiloride did not inhibit uptake of GM1 and GPI-GFP (not shown).

Since modulation of Rac1 activity affected syndecan-4 endocytosis, we examined its effect on FGF2 uptake as well as macropinocytosis as defined by dextran internalization. Transient expression of a dominant negative Rac1 construct significantly inhibited FGF2 endocytosis while a constitutively

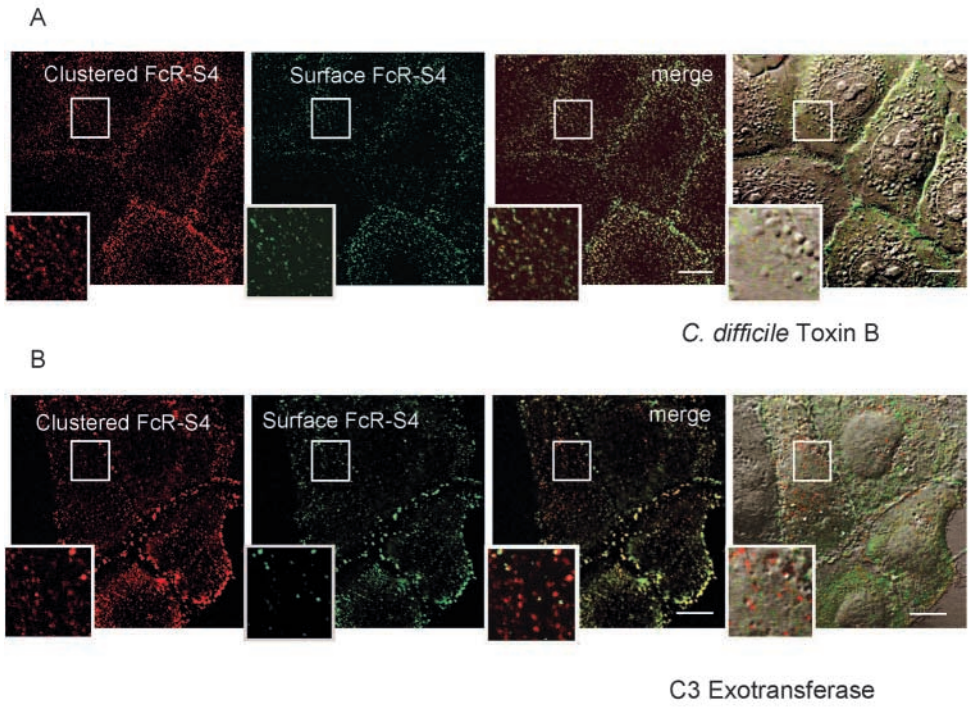


Fig. 7. Role of Rho family GTPases in syndecan-4 endocytosis. The effect of pre-treatment with *C. difficile* toxin B (A) and C3 exotransferase (B) on syndecan-4 endocytosis was examined in antibody-clustered FcR-S4 chimeras. Note that while toxin B treatment completely inhibited FcR-S4 endocytosis, C3 exotransferase had no effect. Scale bars, 10 μm.

active Rac1 increased it (Fig. 10a,b). At the same time neither construct affected dextran uptake (Fig. 5b).

To contrast effects of membrane cholesterol depletion and amiloride and clathrin-dependent uptake of transferrin and clathrin-independent endocytosis of syndecan-4 and to corroborate confocal microscopy data, we used flow cytometry to quantitate these processes. In agreement with the confocal microscopy results, cyclodextrin treatment virtually completely inhibited syndecan-4 uptake while reducing transferrin endocytosis to a lesser degree (Fig. 11). This is consistent with similar published results (Rodal et al., 1999; Subtil et al., 1999). However, the possibility of a yet undefined effect of cyclodextrin on macropinocytosis cannot be excluded. Amiloride also fully inhibited syndecan-4 uptake as expected for a macropinocytic process, while mildly stimulating transferrin entry into cells (Fig. 11).

Finally, to explore whether syndecan-4 clustering affects the rate of macropinocytosis, we measured dextran uptake following antibody clustering of FcR-S4 chimeras, native syndecan-4 or following FGF2 treatment. In all cases, dextran uptake was not affected (not shown) suggesting that syndecan-4 clustering or FGF2 treatment does not stimulate macropinocytosis.

Discussion

The results of this study demonstrate that FGF2 internalization proceeds in a syndecan-4-dependent manner and document the endocytic pathway of syndecan-4 uptake. The pathway is initiated by the cell plasma membrane syndecan-4 clustering that results in Rac1 activation and proceeds in a caveolae-, clathrin- and dynamin-independent manner. Moreover, it is amiloride and Cdc42 sensitive and results in dextran and FGF2 internalization in the syndecan-4-containing vesicles. These data are consistent with the notion that syndecan-4 clustering initiates its endocytosis via activation of Rac1 that allows syndecan-4 to detach from the actin cytoskeleton, enter the macropinocytic pathway and to internalize FGF2.

Receptor endocytosis can proceed in a clathrin-dependent and -independent manner (Conner and Schmid, 2003). While endocytosis of most receptors, including G protein-coupled (Pierce et al., 2000) and tyrosine kinase receptors (Vieira et al., 1996) was originally described as clathrin-dependent, recent studies have clearly shown that clathrin-independent receptor endocytosis plays an equally important role (Nichols and Lippincott-Schwartz, 2001). To date a number of different clathrin-independent internalization pathways have been described that typically involve GTPase dynamin, different Rho GTPases and either caveolae or non-caveolar lipid raft portion of the plasma cell membrane. One such pathway is the internalization of interleukin-2 receptor from the plasma membrane rafts. The process is rapid and requires the GTPase dynamin and a small GTPase RhoA in an activated state (Lamaze et al., 2001). The latter observation is in marked contrast to the clathrin-mediated endocytosis where RhoA and Rac1 act as negative regulators.

An example of another pathway is folate uptake via its receptor, a GPI-anchored protein, that is accomplished in a clathrin, dynamin- and caveolae-independent manner and is regulated by Cdc42 (Sabharanjak et al., 2002). This pathway bears considerable similarity to FGF2/syndecan-4 endocytosis including its origin from the plasma membrane rafts, lack of dependence on dynamin and clathrin and inhibition by a Cdc42 dominant negative.

The major difference between the two processes arises from FGF2/syndecan-4 uptake dependence on activation of Rac1 that has not been reported by the folate/GPI protein endocytosis. The involvement of Rac1 in a non-clathrin-dependent endocytosis has previously been described but it has been limited to fluid phase pinocytosis that seems also to involve Pak1 (Dharmawardhane et al., 2000). Rac1 is also involved in type 1 phagocytosis where it acts in conjunction

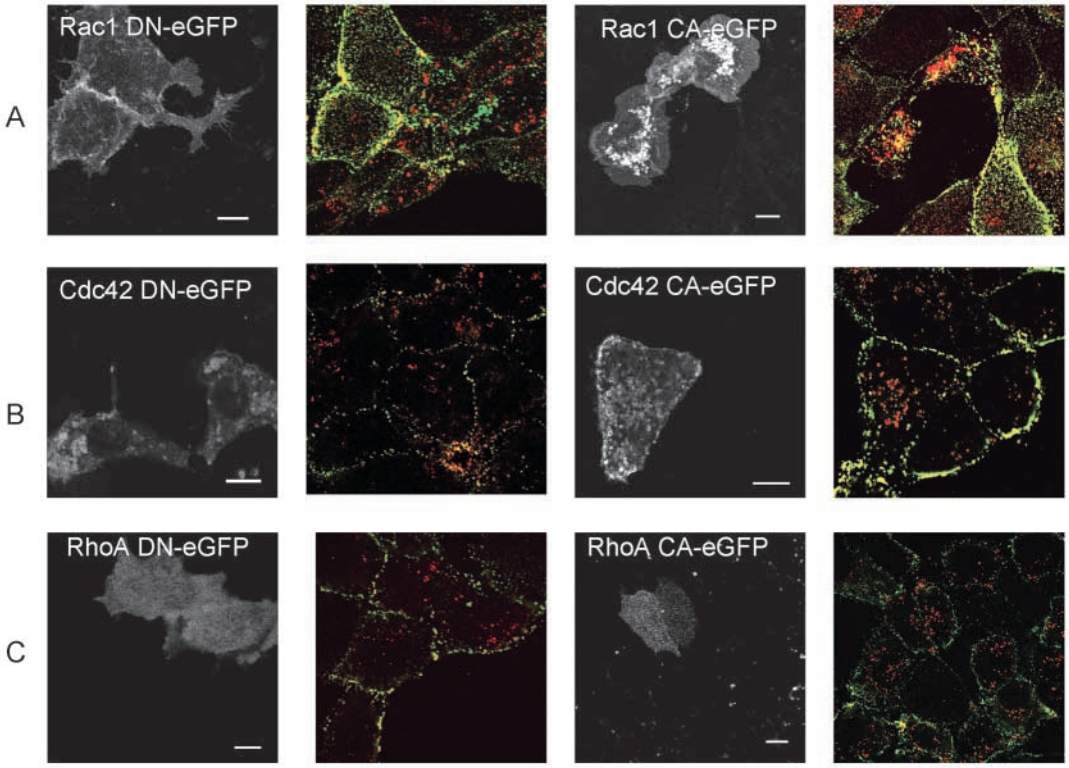


Fig. 8. Specific role of Rho GTPases in syndecan-4 endocytosis. The contributions of Rac1, Cdc42 and RhoA GTPases to syndecan-4 endocytosis were studied in FcR-S4-expressing RFPEC transiently transfected with eGFP-tagged dominant negative and constitutively active constructs. (A) A dominant negative Rac1 completely inhibited (left panels) while a constitutively active Rac1 stimulated (right panels) FcR-S4 endocytosis. (B) The expression of a dominant negative Cdc42 construct (left) somewhat inhibited FcR-S4 endocytosis while a constitutively active (right) Cdc42 constructs had no effect. (C) The expression of dominant negative (left) or constitutively active (right) RhoA constructs had no effect on FcR-S4 endocytosis. CA, constitutively active; DN, dominant negative. Scale bars, 10 μm.

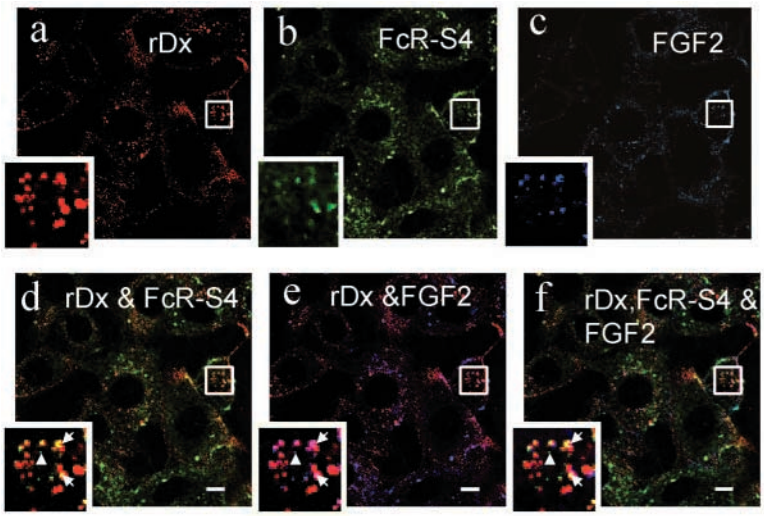
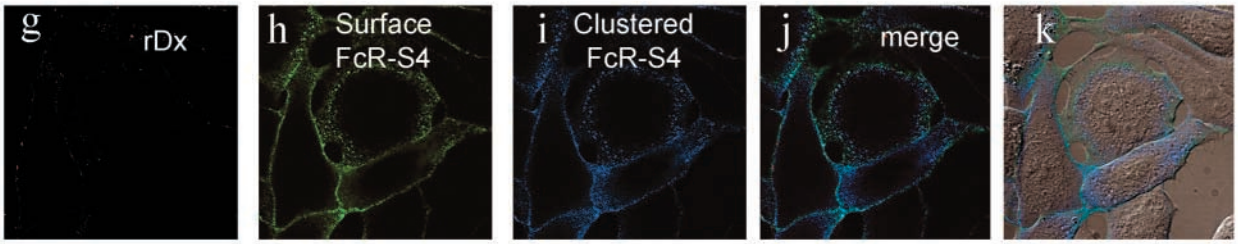


Fig. 9. Syndecan-4 and FGF2 uptake proceed via macropinocytosis. FcR-S4-expressing RFPEC cultured with fluorescent dextran (red, a) were decorated by niIgG (green, b) followed by FGF2 (blue, c) clustering. Merged images demonstrate co-localization of dextran and syndecan-4 (d), dextran and FGF2 (e) and all three signals (f). Amiloride pre-treatment completely inhibited endocytosis of both dextran (g) and FcR-S4 (h-j). (k) The merged image overlaid with the DIC image. Scale bars, 10 μm.



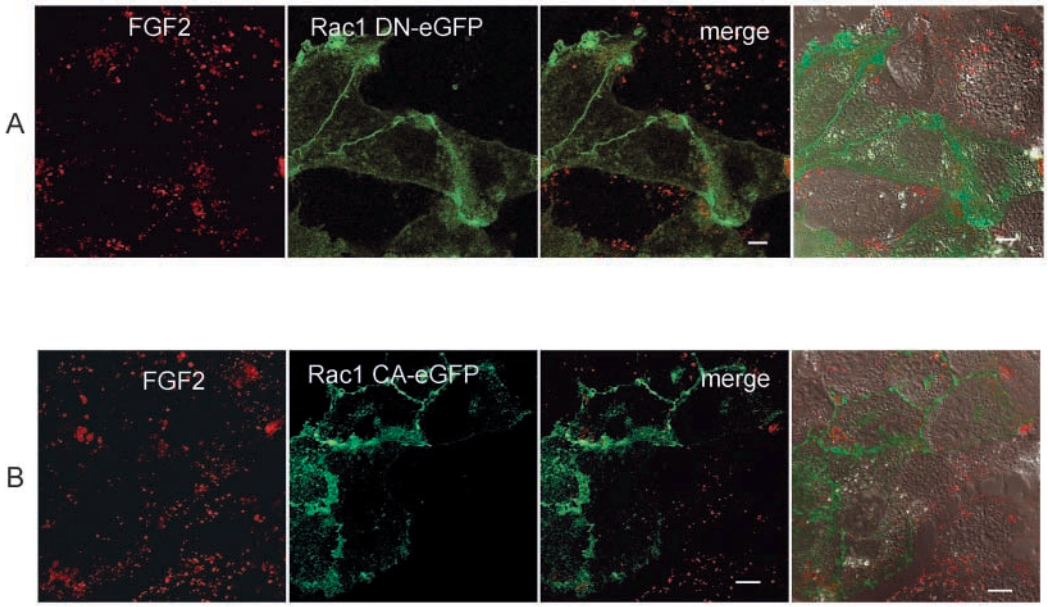


Fig. 10. Rac1 regulates FGF2 endocytosis. The effect of transient expression of Rac1 dominant negative (Rac1 DN-eGFP, A) and constitutively active (Rac1 CA-eGFP, B) constructs on FGF2 endocytosis was examined in RFPEC treated with fluorescently labeled FGF2 (red). Note decreased FGF2 uptake in cells expressing Rac1 DN-eGFP and increased uptake in cells expressing Rac1 CA-eGFP. Scale bars, 10 μm.

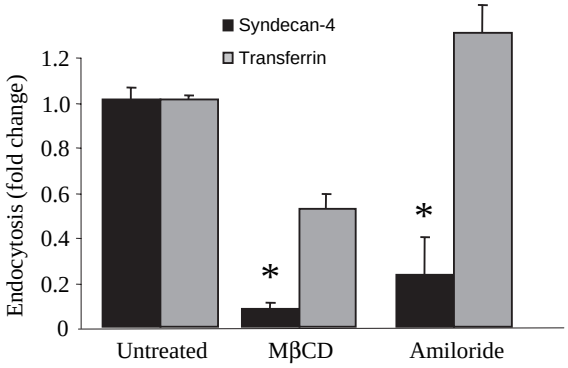


Fig. 11. Flow cytometry analysis of syndecan-4 endocytosis. The effects of membrane cholesterol depletion by treatment with methyl-β-cyclodextrin (MβCD) and amiloride on uptake of transferrin and FcR-syndecan-4 were analyzed using flow cytometry as described in the Materials and Methods. The data are presented as fold change from baseline. MβCD treatment almost fully blocked syndecan uptake while significantly reducing the level of transferrin internalization. Amiloride treatment inhibited syndecan uptake but increased the rate of transferrin endocytosis. *P<0.05 versus baseline.

with Cdc42 (Ellis and Mellor, 2000). However, Rac1 function is very complex as shown by studies in epithelial cells where expression of the dominant negative Rac1 decreased the rates of apical and basolateral endocytosis and inhibited apical recycling of IgA whereas the constitutive active Rac1 mutant had the opposite effect (Jou et al., 2000)

In the case of syndecan-4, Rac1 activation is required for its endocytosis while the activity of RhoA does not affect this process. This conclusion is supported by several observations. First, is the observation that a pan-Rho GTPase inhibitor, *C. difficile* toxin B, inhibited all syndecan-4 uptake while a RhoA specific inhibitor, C3 exotransferase, had no detectable effect on its endocytosis. Second, a dominant negative Rac1 mutant completely inhibited syndecan-4 uptake while a constitutive active Rac1 mutant markedly accelerated it. At the same time, dominant negative and constitutive active forms of RhoA had no effect on syndecan-4 endocytosis. Of note, the time course of Rac1 activation by syndecan-4 oligomerization is in keeping with its above described role in syndecan-4 uptake since its activation precedes the initiation of the core protein internalization and its activity returns to normal when the process is complete. The capacity of syndecan-4 clustering to activate Rac1 is in keeping with the known syndecan-4 oligomerization-dependent activation of PKCα (Horowitz and Simons, 1998; Oh et al., 1997) and the ability of PKC agonist PMA to activate Rac1 and to induce macropinocytosis (Grimmer et al., 2002).

There are several possible explanations for the role of Rac1 in syndecan-4-dependent endocytosis. In quiescent cells plated on fibronectin syndecan-4 participates in formation of focal adhesions and is found in association with the actin cytoskeleton (Saoncella et al., 1999). This linkage to actin may involve recently described syndecan-4 binding to α-actinin (Greene et al., 2003). Prior to clustering, syndecan-4 is absent from the raft plasma membrane domain and it moves there in response to FGF2 or antibody-induced oligomerization (Tkachenko and Simons, 2002). This data is in agreement with the finding that more than 50% of cell surface bound FGF2 is found in lipid rafts (Chu et al., 2004). Such a movement probably requires breaking of the syndecan-4-actin cytoskeleton association, an event accomplished by Rac1 activation. Once translocated to the raft domain, syndecan-4 endocytosis proceeds in a manner similar to that of the folate receptor. This is suggested by co-localization of syndecan-4 and BODIPY-GM1 and GPI-GFP markers of raft region proteins and by the effect of dominant-negative CDC42. This finding is also in agreement with a previously reported co-localization of syndecan-4 and the inactive variant of proaerolysin, a marker of GPI-associated proteins (Tkachenko and Simons, 2002).

The sensitivity of syndecan-4 uptake to inhibition with amiloride and the presence of dextran in syndecan-4-containing vesicles suggest that it proceeds via macropinocytosis, a process that proceeds by formation of

large endocytic vesicles brought about by the closure of lamellopodia at ruffling membrane domains and is usually thought of in the context of bulk fluid uptake. A recent study demonstrated that dynamin 2 is required for PDGF-induced but not constitutive macropinocytosis (Schlunck et al., 2004). This finding suggests that there are at least two different mechanisms of macropinocytic uptakes. This distinction is also emphasized by the fact that while syndecan-4 is associated with GM1 and GFP-GPI on the cells surface and in endosomes they do not follow the same endocytic pathway.

It has been suggested that macropinocytosis may be involved in internalization of large multimeric complexes and may play a role, for example, in viral entry into cells (Liu et al., 2002; Marechal et al., 2001) as well as endocytosis of several endothelial plasma membrane proteins including ICAM-1, PECAM-1 and E-cadherin (Muro et al., 2003; Paterson et al., 2003). Finally, macropinocytosis is an attractive pathway for syndecan internalization given the typical large sizes of its heparan sulfate chains. This role of rafts in recruitment for macropinocytosis may be similar to the recruitment of cholera and anthrax toxins for uptake via the clathrin pathway (Abrami et al., 2001; Shogomori and Futerman, 2001).

FGF2 internalization is an important consequence of syndecan-4 endocytosis. In the cell type under study the majority of FGF2 uptake occurred via the syndecan-4 pathway. The dependence of FGF2 uptake on syndecan-4 endocytosis is in keeping with previously published results that suggested that both FGF1 and FGF2 enter cells via an endocytic process involving heparan sulfate proteoglycans (Citores et al., 1999; Gleizes et al., 1995; Roghani and Moscatelli, 1992). Such proteoglycan-dependent uptake has been localized to uncoated pits, in keeping with our finding of non-clathrin-dependent uptake, and proceed at a relatively slow pace, once again consistent with the kinetics of syndecan-4 internalization observed in this study (Gleizes et al., 1995). The functional significance of syndecan-4-dependent FGF2 internalization has not been established but previous studies have suggested that FGF1 uptake is required for its full mitogenic effect (Grieb and Burgess, 2000).

In summary, we describe macropinocytosis of syndecan-4 triggered by its oligomerization on endothelial cell membranes that results in internalization of the clustering agent, in this case FGF2, and involves both Rac1 and Cdc42 GTPases. The biological function of this pathway is the subject of future studies.

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