Clustering Induces Redistribution of Syndecan-4 Core Protein into Raft Membrane Domains*

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Syndecan-4 is a heparan sulfate-carrying core protein that has been directly implicated in fibroblast growth factor 2 (FGF2) signaling. Recent studies have suggested that many signaling proteins localize to the raft compartment of the plasma cell membrane. To establish whether syndecan-4 is present in the raft compartment, we have studied the distribution of the core protein and an Fc receptor (FcR)-syndecan-4 chimera prior to and following clustering with FGF2 or antibodies. Whereas unclustered syndecan-4 was present predominantly in the non-raft membrane compartment, clustering induced extensive syndecan-4 redistribution to the rafts as demonstrated by the sucrose gradient centrifugation and life confocal microscopy. Although syndecan-4 and caveolin-1 moved in tandem, syndecan-4 was not present in caveolae, a major subset of raft compartments. We conclude that syndecan-4 clustering induces its redistribution to the non-caveolae raft compartment. This process may play an important role in syndecan-4-mediation of FGF2 signaling.

Syndecans, a four-member family of transmembrane proteoglycan core proteins, are found in a wide spectrum of cells and engage in a variety of interactions including binding growth factors, growth factor receptors, matrix proteins such as fibronecrti and vitronectin, ligands, and others with biologically active molecules (1, 2). All syndecans carry both heparan sulfate and chondroitin sulfate chains on their extracellular domain and engage in PDZ (Postsynaptic density 95, Disk large, Zona occludens-1)-dependent interaction via conserved intracellular domains (1, 2). Syndecan-4 is a unique member of the syndecan core protein family. Unlike other syndecans, it possesses a phosphoinositol 4,5-bisphosphate binding site in its cytoplasmic tail that allows it to bind and activate protein kinase Cα (3, 4). Syndecan-4 is found on endothelial cells and is directly involved in the regulation of FGF2-induced cell growth and migration (5).

Although previous studies have shown that syndecan-4 is predominantly found on the basolateral plasma cell membrane and focal adhesions (6, 7), little information is available regarding its association with various membrane subdomains. Recent studies have suggested that raft, lipid-ordered microdomains enriched by cholesterol and sphingolipids, act as platforms for conducting a variety of cellular functions such as vesicular trafficking and signal transduction (8, 9). Therefore, we have conducted this study to determine whether activation of syndecan-4 signaling initiated by its oligomerization on the plasma cell membrane leads to its appearance in the membrane rafts.

Several protein families have been reported to modify lipid rafts structurally and functionally. These include integral membrane proteins such as caveolins and flotillins, exoplasmic glycosylphosphatidylinositol (GPI)1-linked proteins such as Thy-1 and alkaline phosphatase, and receptor tyrosine kinases among others (9). Caveolin integration into the microenvironment of a lipid raft leads to raft invagination and formation of caveolae (9–11). Caveolae are formed in many cell types including endothelial cells (12), and they may play a role in modulation of cell signaling. One hypothesis suggests that interactions of caveolins with signaling molecules regulate their activation status.

Because FGF2-induced syndecan-4-dependent signaling requires the presence of heparan sulfate chains on core protein extracellular domain (13), it is reasonable to hypothesize that FGF2 induces aggregation of syndecan-4 complexes in the plasma cell membrane. However, because FGF2 induces a number of other signaling events that may affect syndecan-4 movement in the plasma cell membrane in manner independent of oligomerization of its extracellular domains, we linked the extracellular domain of the human Fc receptor (FcR)-Ia to the transmembrane/cytoplasmic domains of syndecan-4. The resultant chimera can then be aggregated with an immunoglobulin, mimicking FGF2-induced syndecan-4 oligomerization. We found that aggregation of syndecan-4 cores leads to a shift of syndecan-4 complexes from the non-raft to raft microdomains of the plasma cell membrane. Although syndecan-4 motion was synchronous with movements of caveolin-1, syndecan-4 was not present in the caveolae portion of the plasma membrane rafts.

MATERIALS AND METHODS

Antibodies and Reagents—Polyclonal rabbit antisera against cytoplasmic and extracellular domains of syndecan-4 were described elsewhere (14). Polyclonal chicken IgY against extracellular domains of syndecan-4 was produced (Aves Laboratories) using the same peptide as previously used for generation in rabbits (14). Anti-FcR (CD64) monoclonal antibody was purchased from Abcam (Cambridge, United Kingdom). Cy-3 and biotin-SP-conjugated non-immune human IgG, Cy-3-conjugated goat anti-human F(ab′)2 fragment, Cy-5-conjugated streptavidin, and goat anti-chicken F(ab′)2 fragment were purchased from Rockland Immunochemicals, Inc. (Gilbertsvile, PA). Alexa-488-conjugated inactive variant of the protein proaerolysin; HRP, horseradish peroxidase; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; RFPEC, Rat fad pad endothelial cells; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FGF2, fibroblast growth factor 2.

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; FcR, Fc receptor Ia CD64; FcR-II, Fc receptor II CD32; FcRIII, Fc receptor III CD16; FLAER, Alexa-488-conjugated inactive variant of the protein proaerolysin; HRP, horseradish peroxidase; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; RFPEC, Rat fad pad endothelial cells; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; FGF2, fibroblast growth factor 2.

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from Jackson ImmunoResearch. Secondary antibodies conjugated to Alexa-594 and a nuclear stain ToPro3 were from Molecular Probes. Alexa-488-conjugated inactive variant of the protein procerulysin (FLAER) was purchased from Protox Biotech (Victoria, British Columbia, Canada). Secondary antibodies conjugated to horseradish peroxidase were labeled from Vector Laboratories, and immobilized streptavidin and HRP-conjugated streptavidin were from Pierce. HRP-conjugated cholera toxin β subunit was purchased from Sigma.

cDNA Constructs and Transfection—Fc receptor-syndecan-4 chimera (FcR-S4) was constructed by linking the ectoplasmic domain (amino acids 1-288) of human Fc receptor la (CD64) cDNA and the transmembrane and cytoplasmic domains of rat syndecan-4 (amino acids 150-202). The chimera and the full-length syndecan-4 construct were inserted into pCR 3.1-Uni expression vector (Invitrogen). Cavelin-1-EGFP cDNA construct (15) was a gift of Dr. L. Pelkmans (Institute of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland).

RFPEC cells were cultured in DMEM medium (Invitrogen) as described previously (16). Stable expression of FcR-S4 chimera was achieved by transfecting the cDNA construct into wild-type RFPEC using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were selected for neomycin resistance (0.4 mg/ml Geneticin, Invitrogen), and pooled populations were used for all studies. To enrich each pool with cells having high expression levels of FcR-S4 protein, cells were subjected to two rounds of fluorescence-activated sorting (see below) to select cells in the top 10% of the population.

Transient expression of cavelin-1-EGFP and pEGFP-N1 (CLONTECH) in RFPEC was achieved by transfection using LipofectAMINE 2000. Cells were used within 48-72 h after transfection.

Fluorescence-assisted Cell Sorting—Cells were dissociated from plates using non-enzymatic solution (Sigma) and labeled for 20 min with fluorescein isothiocyanate-conjugated non-immune IgG (0.1 μg/ml, Rockland) in Dulbecco’s phosphate-buffered saline with 1% bovine serum albumin (BSA), thus specifically detecting the transfected Fc receptor that is not endogenously expressed in endothelial cells. To detect endogenous syndecan-4, cells were incubated with 1 μg/ml chicken antibody against extracellular domain of syndecan-4 for 20 min at room temperature, washed twice in 1% BSA-DMEM, and then incubated with a secondary antibody (Cy-5-conjugated Fab’2 fragment, 1 μg/ml) for 20 min at room temperature followed by another wash with 1% BSA-DMEM. Cell sorting was carried out on a MoFlo sorter (cytometry) with a FACScan (BD Pharmingen) and analyzed using WinMDI version 2.8 ( Scripps Institute, La Jolla, CA) software.

Cell Surface Biotinylation—The cells were washed twice with ice-cold DMEM and incubated with 0.5% BSA-DMEM containing a 1:50 dilution of 1% Triton X-100 in MNE buffer (150 mM NaCl, 2 mM EDTA, 25 mM, pH 6.5) with 1% Triton X-100 and protease inhibitor mixture in TNE buffer for 20 min at 4 °C. The supernatant was transferred to the fresh tube. To recover membrane rafts proteins, Triton X-100 pellet was resuspended in 100 μl of pellet lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1% Triton X-100) and centrifuged for 2 min at 4 °C. The supernatant was then analyzed by immunoprecipitation, SDS-PAGE, and immunoblotting. Glycosaminoglycan chains were digested as described previously (4).

Plasma membrane cholesterol depletion was accomplished by pre-treatment of cultured cells with β-cyclodextrin (10 μM), which removes unesterified cholesterol from the plasma membrane (19) for 30 min at 37 °C immediately followed by chilling the cells to 4 °C.

Immunoprecipitation, Immunoblotting, and Dot-blotting—Immunoprecipitation of syndecan-4 from cell lysates was carried out by incubation of 500 μl of total cell lysates with 10 μl of anti-syndecan-4 cytoplasmic tail antisera in the presence of protein G/protein A-agarose (30 μl). Prior to immunoblotting, glycosaminoglycan chains were digested as described previously (4). The immunoprecipitated material was then subjected to SDS-PAGE and transferred to the polyvinylidene difluoride membrane (Millipore). Syndecan-4 detection was performed using HRP-conjugated streptavidin (0.2 μg/ml) for cell surface-biotinylated samples or by anti-syndecan-4 antisera (1 μl/ml) followed by incubation with a secondary HRP-conjugated goat antibody specific for the primary anti-syndecan-4 antibody.

Immunoprecipitation of syndecan-4 from sucrose gradient fractions was performed in the similar manner. The precipitated material was then Dot-blotted on the nitrocellulose membrane (Schleicher & Schuell), and syndecan-4 was detected by HRP-conjugated streptavidin.

For GM1 sphingolipid detection in sucrose gradient fractions, 100 μl of each fraction were applied to the nitrocellulose membrane, and GM1 was then detected using HRP-conjugated cholera toxin subunit β (0.2 μg/ml). GM1 detection in Triton X-100-soluble and insoluble fractions was carried out by blotting 20 or 200 μl of each fraction on the nitrocellulose filter, which was then probed with HRP-conjugated cholera toxin subunit β (0.2 μg/ml). All blots were developed using enhanced SuperSignal West Pico chemiluminescent substrate (Pierce).

Live Fluorescent Microscopy—For live fluorescent microscopy, cells were grown on gelatin 0.1% phosphate-buffered saline-coated coverslips (Fisher) to confluence. Staining for FcR-S4 was done using biotinylated non-immune IgG, and the biotin label was then visualized by incubation with Cy-5-conjugated streptavidin (1 μg/ml) for 30 min at 37 °C. Cells were then blocked and permeabilized with 10 μg/ml of 0.2% FLAER at 37 °C for 1 h. FLAER binds selectively to cholesterol anchors (20).

Coverslips were removed from the staining solution and mounted on a microscope slide. All microscopy imaging was done using Bio-Rad MRC-1024 krypton/argon laser confocal system microscope with x63 lens by Zeiss. Time-lapse microscopy was carried out at 20 °C. Image analysis was done using Adobe Photoshop 6.0 (Adobe Systems Inc.) and ImageJ software (NIH).

RESULTS

To study the distribution of syndecan-4 core protein in the plasma cell membrane and examine the effect of ligand clustering on its spatial localization, we linked the cytoplasmic and the transmembrane domains of syndecan-4 to the extracellular domain of the Fc receptor. To demonstrate the expression of the FcR-S4 chimera on the cell surface, FcR-S4 construct and empty vector-transfected cells were subjected to fluorescence-assisted cell sorting using fluorescein isothiocyanate-labeled non-immune IgG that specifically recognizes Fc receptor and anti-syndecan-4 ectoplasmic domain antibodies. FcR expres-

sion was noted only on the FcR-S4 construct-transfected cells (Fig. 1A). Furthermore, the expression of the native syndecan-4 was not affected by FcR-S4 expression (Fig. 1A). To demonstrate that the cytoplasmic tails of FcR-S4 chimeras interact with native syndecan-4 proteins, RFPEC cells expressing FcR-S4 construct were decorated with non-immune IgG. The clustering of FcR-syndecan-4 complexes was then performed with the anti-IgG antibody. Clustered and unclustered RFPEC were then lysed and subjected to immunoprecipitation with the antibody against the ectoplasmic domain of syndecan-4 fol-

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raft formation by depleting membrane cholesterol by treating depends on high local cholesterol ester content, we disrupted insoluble fraction. Because the formation of membrane rafts distribution of soluble fraction (Fig. 2). Antibody clustering of FcR-S4 chimeras were subjected to immunoprecipitation with the anti-ectoplasmic domain syndecan-4 antibody followed by Western blotting of the precipitate with anti-FcR (CD64) antibody. Note the presence of bands corresponding to FcR-S4-syndecan-4 monomer and dimer.

The expression of the native syndecan-4 is the same in vector-transfected (orange) and FcR-S4-transfected (blue) cells. FcR-S4 forms complexes with native syndecan-4. Total cell lysates from cells transfected with FcR-S4 construct prior to (-) or after (+) antibody clustering of FcR-S4 chimeras were subjected to immunoprecipitation with the anti-ectoplasmic domain syndecan-4 antibody followed by Western blotting with an anti-FcR (CD64) antibody.

Clustering Induces Redistribution of Syndecan-4 Core Protein

The distribution of syndecan-4 in the plasma cell membrane was examined by subjecting whole cells lysates of RFPEC-expressing FcR-S4 construct to sucrose density gradient centrifugation. Blotting of the syndecan-4 antibody-immunoprecipitated material from various sucrose gradient fractions demonstrated the core protein presence in both heavy (40% sucrose) and light (10–15% sucrose) fractions (Fig. 2A, right panel). The lighter fractions contain plasma membrane raft proteins as shown by blotting with HRP-conjugated cholera toxin subunit β, which specifically binds to the raft marker GM1 (Fig. 2A, left panel). In the absence of antibody clustering, most of syndecan-4 appeared in the non-raft membrane fractions. Antibody clustering of FcR-S4 chimeras induced a pronounced shift toward the raft-containing fractions (Fig. 2A, right panel). The specificity of HRP-conjugated cholera toxin subunit β-GM1 binding for the detection of raft fractions of the membrane was tested by Dot-blot analysis of Triton X-100-soluble and insoluble portion of the plasma cell membrane (Fig. 2B). As expected, only the Triton X-100-insoluble fraction was labeled with the cholera toxin.

To further explore the relationship between clustering and syndecan-4 redistribution into the raft compartment, we studied syndecan-4 appearance in the Triton X-100-insoluble (raft) and soluble (non-raft) fractions of plasma cell membranes. Equal numbers of FcR-S4 expressing cells were subjected to lysis in Triton X-100 as described under "Materials and Methods." The Triton X-100-soluble and insoluble fractions were then immunoprecipitated with anti-syndecan-4 antibody, separated on SDS-PAGE, and subjected to Western blotting with HRP-streptavidin. Prior to antibody clustering of FcR-S4 chimeras, essentially all of syndecan-4 was in the Triton X-100-soluble fraction (Fig. 2C). Antibody clustering induced redistribution of ~50% cell surface syndecan-4 to the Triton X-100-insoluble fraction. Because the formation of membrane rafts depends on high local cholesterol ester content, we disrupted raft formation by depleting membrane cholesterol by treating cells with β-cyclodextrin. Such cholesterol depletion almost fully prevented clustering-induced syndecan-4 redistribution into the rafts compartment (Fig. 2C).

Because clustering of the native syndecan-4 with its natural ligands such as FGF2 may potentially have a different effect on its plasma membrane dispersal than antibody clustering of FcR-S4 chimeras, we have examined the effect of FGF2 treatment on syndecan-4 distribution in RFPEC cells. Similar to the FcR-S4 chimera antibody clustering studies, FGF2 induced a significant redistribution of syndecan-4 from Triton X-100-soluble to insoluble fraction (Fig. 2D).

To confirm localization of clustered syndecan-4 to plasma membrane rafts, we used vital confocal microscopy with anti-syndecan-4 antibody and the GPI-anchor marker FLAER in RFPEC. Following antibody clustering of FcR-S4 chimeras, FLAER imaging demonstrated prominent raft clusters on the apical plasma cell membrane (Fig. 3, top left panel). Staining with a labeled non-immune IgG that detects FcR-S4 chimeras demonstrated equally prominent FcR-S4 clusters on the apical plasma cell membrane (Fig. 3, top right panel). The merged image demonstrates that essentially all FcR-S4 chimeras overlapped with FLAER labeling (Fig. 3, top right panel), consistent with syndecan-4 presence in the FLAER-labeled rafts. The specificity of staining was confirmed by FLAER and non-immune IgG labeling of the vector-transfected RFPEC (Fig. 3, bottom panels) that demonstrated the absence of FcR-S4 signal.

Caveolae constitute a distinct subpopulation of cholesterol-rich rafts. To see whether syndecan-4 is present in caveolae, we generated RFPEC cells expressing EGFP-tagged caveolin-1. Live confocal microscopy showed no co-localization between syndecan-4 and caveolin-1 (Fig. 4A) and coordinated the movement of the two proteins (movie file). To explore the effect of syndecan-4 clustering on its association with caveolae, we used time-lapse microscopy to visualize syndecan-4 movement in caveolin-1-EGFP-expressing RFPEC cells. The reconstruction of time stacks of syndecan-4-caveolin-1 images in the x y plane demonstrated that although both proteins moved together, they did not localize to the same cellular compartment.
Western blots with an anti-caveolin-1 antibody failed to detect its presence among proteins present in the syndecan-4 immunoprecipitations (data not shown). To control for the effect of EGFP expression on the cellular distribution of caveolin-1-EGFP chimeric protein, RFPEC were transiently transfected with an EGFP construct. A visualization of EGFP demonstrated diffuse distribution of the protein in the cytoplasm (Fig. 4C), quite distinct from the appearance of caveolin-1-EGFP protein. Syndecan-4 staining is present along the cell border (Fig. 4C).
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DISCUSSION

Whereas syndecans have largely been considered structural proteins, recent data suggest that they play a variety of roles including lipoprotein uptake (21), cell adhesion (6), and regulation of FGF2 signaling (5, 22). However, the molecular events involved in these activities are not well understood. Previous studies have shown that in Chinese hamster ovary cells, syndecan-1 is found upon clustering in the Triton X-100-insoluble regions of the membrane (23) and that this event may play a role in syndecan-1-mediated lipid endocytosis (21, 24). Although syndecan-1 and syndecan-4 share significant homologies in the transmembrane and cytoplasmic regions domains, which are the regions thought to play a role in clustering-induced aggregation in membrane rafts, there are also significant differences. Syndecan-4 is a unique member of the syndecan core protein family because of its ability to engage in FGF2 signal transduction. Furthermore, no data have previously been available regarding the effect of FGF2 binding on syndecan-4 clustering and the occurrence of this event in polarized endothelial cells.

Because FGF2 treatment induces a number of cellular events that can potentially affect membrane syndecan-4 trafficking, we employed FcR-syndecan-4 chimeras to study the effect of syndecan clustering on its localization in the plasma cell membrane. The use of extracellular domain of Fc receptor is particularly convenient, because Fc receptor is not expressed in endothelial cells and non-immune IgG can be used to decorate cells expressing Fc receptor. The chimera protein behaves in a mode analogous to the native syndecan-4. It is present on the cell surface, it forms complexes with the native syndecan-4, and its expression does not affect the expression of the native protein. Therefore, FcR-S4 clustering behavior is representative of ligand clustering of the native syndecan-4.

We find that in quiescent endothelial cells, FcR-S4 chimeras as well as native syndecan-4 are present mostly in the non-raft fraction of the membrane and that the aggregation of FcR-S4 chimeras with antibodies or syndecan-4 with FGF2 leads to a rapid redistribution of the proteins to the membrane rafts. Several independent pieces of data are consistent with this interpretation including sucrase gradients, Western blotting of various membrane fractions, and life confocal microscopy.

Two principle raft subdomains are caveolae-containing and caveolae-free rafts. Interestingly, syndecan-4 did not co-localize with caveolin-1, suggesting that it was not present in the caveolae but rather in the non-caveolae rafts. The absence of caveolin-1 in the syndecan-4-immunoprecipitated material further argues against direct interaction between these proteins. At the same time, the clustering of FcR-S4 chimeras led to close apposition, but apparently not the binding, of syndecan-4 and caveolae and a complex-coordinated movement of both proteins.

Although we have not examined which portion of the syndecan-4 molecule mediates its association with plasma membrane rafts, the transmembrane domain and the immediately adjacent highly cationic cytoplasmic domain are the likely candidates by association with syndecan-1. To note, the presence of the phosphoinositol 4,5-bisphosphate binding domain in syndecan-4 (absent in syndecan-1) does not inhibit its localization to the raft subdomain. The presence of plasma membrane cholesterol is clearly required for this event as its depletion led to a virtually complete loss of syndecan-4 enrichment in Triton X-100-insoluble regions.

Although syndecan-4 clustering with its signaling agonist FGF2 induces its redistribution to non-caveolae rafts, the functional significance of this event is unclear. One possibility is that this serves to bring syndecan-4 in close contact with FGF receptors. Because FGF receptor-dependent activation of a protein phosphatase is needed for the activation of syndecan-4 signaling (16), syndecan-4 movement into rafts would serve to facilitate this connection. Alternatively, rafts localization of syndecan-4 complexes may bring it in close contact with other signaling complexes, thereby promoting cross-talk among vari-
ious receptor pathways.

In summary, ligand-dependent clustering of syndecan-4 in primary endothelial cells leads to its concentration in non-caveolae rafts. This event may play a role in the regulation of syndecan-4 signaling.

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