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Shedding of Cell Membrane-Bound Proteoglycans

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Abstract

Membrane-bound proteoglycans function primarily as coreceptors for many glycosaminoglycan (GAG)-binding ligands at the cell surface. The majority of membrane-bound proteoglycans can also function as soluble autocrine or paracrine effectors as their extracellular domains, replete with all GAG chains, are enzymatically cleaved and released from the cell surface by ectodomain shedding. In particular, the ectodomain shedding of syndecans, a major family of cell surface heparan sulfate proteoglycans, is an important posttranslational mechanism that modulates diverse pathophysiological processes. Syndecan shedding is a tightly controlled process that regulates the onset, progression, and resolution of various infectious and noninfectious inflammatory diseases. This review describes methods to induce and measure the shedding of cell membrane-bound proteoglycans, focusing on syndecan shedding as a prototypic example.

Keywords

Syndecan; Glypican; Heparan sulfate proteoglycan; Sheddase; Matrix metalloproteinase; ADAM; Inflammation; Infection; Host defense

1. Introduction

All nucleated mammalian cells express at least one membrane-bound proteoglycan on their cell surface. Membrane-bound proteoglycans include the syndecan and glypican families with four and six members in mammals, respectively, NG2 (AN2 in mice), beta-glycan, thrombomodulin, $\alpha 5\beta 1$ integrin, neuropilin-1, and CD44 (1–3). Core proteins of syndecans, NG2, CD44, betaglycan, neuropilin-1, $\alpha 5\beta 1$ integrin, and thrombomodulin are type I trans-membrane proteins, whereas core proteins of glypicans are attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor. Core proteins of membrane-bound proteoglycans have discrete glycosaminoglycan (GAG) attachment sites. Heparan sulfate (HS) chains are attached distal to the plasma membrane on all syndecans and chondroitin sulfate (CS) chains are also attached proximal to the cell surface on some syndecans (e.g., syndecan-1). GAG attachment sites in NG2 (CS) and CD44 (HS/CS) are located in the middle portion of the core protein, whereas those of glypicans (HS), thrombomodulin (CS), and betaglycan (HS/CS) are proximal to the cell surface.

On the cell surface, membrane-bound proteoglycans function primarily as a coreceptor that catalyzes the encounter between ligands and their respective signaling receptors (1–3). Membrane-bound proteoglycans can also function as soluble proteoglycans since the intact extracellular domain can be released from the cell surface by an enzymatic cleavage mechanism known as ectodomain shedding. Ectodomain shedding is of paramount importance to membrane-bound proteoglycan biology because it both rapidly changes the surface phenotype of affected cells by reducing the amount of ligand-binding GAGs and generating soluble proteoglycan ectodomains replete with all their GAGs that can function

as autocrine or paracrine effectors. Syndecans and CD44 are shed by metalloproteinases, whereas glypicans are released from the cell surface by cleavage of their GPI anchor by phospholipases. Other surface proteoglycans are also found in soluble form, but how they are released from the cell surface is not known.

The underlying mechanisms of syndecan-1 shedding have been extensively studied. Syndecan-1 shedding is a highly regulated process that is stimulated *in vitro* by several inflammatory factors, and *in vivo* under certain pathological conditions (1, 4). Agonists of syndecan-1 shedding include growth factors, stress-related agonists, heparanase, and bacterial virulence factors, among others (see Table 1). The majority of these agonists enhance metalloproteinase-mediated syndecan-1 shedding at the cell surface in a protein tyrosine kinase-dependent manner. However, several metalloproteinases can shed syndecan-1 ectodomains (see Table 1), suggesting that the shedding agonists and the signaling pathways that they activate tightly control syndecan-1 shedding.

Accumulating evidence suggests that the shedding of membrane-bound proteoglycans is an important posttranslational mechanism that regulates pathophysiological processes. For example, syndecan-1 ectodomains are elevated in blood of patients with sepsis (5, 6), ischemia-reperfusion injury (7), graft-versus-host disease (8), and various cancers (9, 10), among other fluids from injured, inflamed, or cancerous tissues (11–13). Further, mouse studies have shown that the inflammatory response to toxins, chemicals, allergens, and pathogens is dysregulated in the absence of syndecan-1 or when its shedding is inhibited (14–19), suggesting that syndecan-1 shedding is mobilized to assure the adequate and correct functioning of inflammation. Increased levels of CD44 ectodomains are also detected in bronchoalveolar lavage fluids of patients with eosinophilic pneumonia (20), and the inflammatory response in *Escherichia coli*-induced pneumonia is amplified in CD44 null mice compared to wild-type mice (21). Together, these data suggest that the shedding of membrane-bound proteoglycans may be a critical mechanism in the pathogenesis of a broad range of infectious and noninfectious diseases, and underscore the importance of developing means to precisely measure and analyze the shedding of membrane-bound proteoglycans. Dot immunoblotting or ELISA is used to measure levels of shed proteoglycan ectodomains. A concomitant decrease in levels of cell surface proteoglycans is measured by dot immunoblotting or FACS. Each of these methods is described below in detail.

2. Materials

2.1. Measurement of Syndecan Ectodomains by Dot Immunoblotting

1. Cells: Normal murine mammary gland (NMuMG) epithelial cell is the standard cell type for syndecan shedding assays because they express syndecan-1, -2, and -4 abundantly and syndecan-3 weakly (22), and NMuMG cells have been demonstrated to respond to the majority of syndecan shedding agonists (23–28). For shedding assays, culture NMuMG cells to confluency or 1-day post confluency in 96-well plates (see Note ¹). Other cell types that have been tested in shedding assays include mouse C127 mammary gland epithelial cells (27), SV40-transformed endothelial cells (24, 29), P3X63 myeloma cells (24), NIH3T3 fibroblasts (27) and LA4 lung epithelial cells (27), and human A549 lung epithelial cells (25), HSAEC small airway epithelial cells (28), A431 epidermal cells (25), CAG myeloma cells (30), and ARH-77 myeloma cells transformed without mouse syndecan-1 (30).

¹Use larger culture vessels when using cell types expressing lower levels of syndecans relative to NMuMG cells. For example, LA4 and NIH3T3 cells should be cultured in 24-well plates and A549 cells should be cultured in 48-well plates to confluency and tested in shedding assays.

2. NMuMG cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% FBS and 10 µg/mL insulin.
3. Trypsin-EDTA: Premade 0.25% trypsin and 2.21 mM ethylenediamine tetraacetic acid (EDTA) in Hank's Balanced Salt Solution (HBSS).
4. Shedding agonists: Phorbol 12-myristate 13-acetate (PMA) is dissolved at 10 mM in dimethyl sulfoxide (DMSO) and stored in aliquots at −80°C. Other commercially available shedding agonists and conditions include EGF (Peprotech, Rocky Hill, NJ), C2 ceramide (VWR, West Chester, PA), thrombin (EMD Chemicals, Gibbstown, NJ), TNFα (Peprotech), *Staphylococcus aureus* α- and β-toxin (Toxin Technology, Sarasota, FL), hyperosmolarity (e.g., 600 mOsm NaCl for 2 h), and heat shock (e.g., 45°C for 1 h) (see Table 1).
5. Primary antibodies: 281–2 rat anti-mouse syndecan-1 ectodomain monoclonal antibody (BD Biosciences, San Jose, CA) and Ky8.2 rat anti-mouse syndecan-4 ectodomain monoclonal antibody (BD Biosciences). Several polyclonal antibodies for mouse syndecan-1 and -4, and several monoclonal and polyclonal antibodies for other mouse and human syndecans are also available commercially from several sources. Based on our experience, we recommend using 281–2 and Ky8.2 antibodies for mouse syndecan-1 and -4 studies, and B-B4 or BA-38 mouse anti-human syndecan-1 monoclonal antibodies (AbD Serotec, Raleigh, NC; Abcam, Cambridge, MA) and 5G9 mouse anti-human syndecan-4 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for human syndecan-1 and -4 studies.
6. Secondary antibodies: Horse radish peroxidase (HRP)-conjugated antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). We recommend using pre-adsorbed secondary antibodies to minimize background.
7. Purified syndecan standards: Purified syndecan-1 and -4 ectodomains. Native syndecan-1 and -4 ectodomains can be rapidly purified from the culture supernatant of NMuMG cells by DEAE ion exchange chromatography and 281–2 or Ky8.2 affinity chromatography. The typical yield is 5–10 µg from 1 L of NMuMG cell culture supernatant.
8. Buffers and solutions:
 - a. Acetate buffered saline with Tween-20 (ABS-T): 50 mM sodium acetate (NaOAc, pH 4.5), 150 mM NaCl, and 0.1% (v/v) Tween-20 (see Note ²).
 - b. Tris-buffered saline (TBS): 50 mM Tris, pH 7.5, and 150 mM NaCl.
 - c. Blocking buffer 1: TBS containing 10% (w/v) nonfat dry milk from VWR or Fisher Scientific (see Note ³).
 - d. Blocking buffer 2: TBS with 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20.
9. Blotting paper: Whatman 3MM paper (VWR or Fisher Scientific). Precut the 3MM paper with a paper cutter to fit the size of the dot blotting apparatus. Handle with gloves to avoid contamination.

²Stock solutions of 1 M sodium acetate, pH 4.5, and 5 M NaCl can be used to make ABS-T. Use acetic acid to adjust pH when making 1 M sodium acetate. Tween-20 is viscous so cut the tip of the pipet tip with scissors or use a large orifice tip (VWR Signature Genomic Pipet Tips, VWR) to aliquot this detergent from stock bottles.

³Milk-containing products are easily contaminated and should be made fresh on the day of use.

10. Cationic nylon membrane: Immobilon Ny+ (Millipore, Billerica, MA). Precut the Immobilon Ny+ membrane with a paper cutter to fit the size of the dot blotting apparatus. Handle with gloves to avoid contamination.
11. Dot blot apparatus: Whatman Minifold I Blotting System (VWR).
12. ECL (enhanced chemiluminescence) development reagent (Pierce Chemical, Rockford, IL).
13. Image analysis system: LAS-4000 Multifunctional Imaging Analysis System (Fujifilm, Stamford, CT).
14. X-ray film: Kodak Biomax MR-1 film (VWR or Fisher Scientific).

2.2. Measurement of Cell Surface Syndecan-1 by Mild Trypsinization and Dot Immunoblotting

1. TBS with 0.5 mM EDTA (TBS/EDTA): Dilute 500 mM stock solution of EDTA, pH 8, in TBS (see Note ⁴).
2. TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin: TPCK-treated trypsin (Sigma-Aldrich) is dissolved at 10 µg/mL in TBS/EDTA. Trypsin is autolytic so use freshly dissolved enzyme for best results.
3. Soybean trypsin inhibitor: Soybean trypsin inhibitor (Sigma-Aldrich) is dissolved at 100 µg/mL in TBS/EDTA. Concentrated stock solution of 10 mg/mL can be stored at -80°C.
4. Materials for dot immunoblotting: As described in Subheading 2.1.

2.3. Measurement of Cell Surface Syndecans by FACS

1. Cells: Culture NMuMG cells to confluency or 1 day post-confluency in 6-well plates (BD Falcon Multiwell Flat-Bottom Plates with Lids from VWR).
2. Antibodies: 281-2 anti-mouse syndecan-1 ectodomain antibody; Ky8.2 anti-mouse syndecan-4 ectodomain antibody; and Alexa fluorophore-conjugated secondary antibodies (Invitrogen).
3. Phosphate-buffered saline (PBS).
4. Bovine serum albumin (BSA): BSA powder dissolved fresh to 1% (w/v) in PBS.
5. Paraformaldehyde (PFA): 16% stock ampules (Electron Microscopy Science, Hatfield, PA) diluted to 2% in PBS.
6. Flow cytometer: We use Accuri C6 Flow Cytometer (Accuri Cytometers, Ann Arbor, MI) but most flow cytometers available in individual labs and institutional core programs will suffice.

2.4. Stripping and Reprobing Blots

1. Stripping buffer: Restore Western Blot Stripping Buffer from Pierce Chemical (see Note ⁵).

⁴EDTA stock solution can be prepared in-house. Dissolve EDTA disodium salt to 500 mM in deionized water. Must adjust to pH 8 while stirring to dissolve EDTA.

⁵Stripping buffer can also be prepared in-house. Prepare a stock solution of 62.5 mM Tris, pH 6.7, 2% (w/v) SDS and store at room temperature. Warm to 60°C and add β-mercaptoethanol to 100 mM. Incubate the membrane in the home-made stripping buffer for 30 min at 60°C to strip antibodies. Follow procedures of the commercial stripping buffer in Subheading 3.4 starting at step 5 for re-blotting with a different antibody.

2. Buffers and solutions:
 - a. TBS.
 - b. Blocking buffer 1: TBS containing 10% (w/v) nonfat dry milk.
 - c. Blocking buffer 2: TBS containing 1% (w/v) nonfat dry milk and 0.1% Tween-20 as in Subheading 2.1.
3. Materials for reprobing: As described in Subheading 2.1.

3. Methods

Syndecan ectodomains migrate as a broad smear when fractionated by SDS-PAGE due to the heterogeneous GAG chains, which makes precise quantification of syndecan ectodomains by Western immunoblotting difficult. Thus, syndecan ectodomains in cell and tissue samples are measured by dot immunoblotting or ELISA. Human syndecan-1 and -4 ELISA kits are available from several commercial sources (e.g., American Research Products, Belmont, MA; Cell Sciences, Canton, MA, IBL America, Minneapolis, MN; Abcam, Cambridge, MA), but commercial ELISA kits are currently not available for other human syndecans and mouse syndecans. Further, the commercial ELISA kits use recombinant syndecans as standards. In our experience, the anti-syndecan monoclonal antibodies used in the majority of these ELISA kits tend to react less efficiently with recombinant syndecans compared to native purified syndecans. Thus, it is likely that levels of syndecan ectodomains in biological samples are overestimated in the commercial ELISA kits. Another disadvantage of the commercially available ELISA kits is that they are expensive. Regardless, the ELISA kits are suitable for rapid qualitative measurements of syndecan ectodomains and their ease of use is similar to that of the dot immunoblotting method described below.

It is also important to measure cell surface syndecan levels when assessing syndecan shedding. Ectodomain shedding is a posttranslational mechanism that rapidly decreases the amount of surface molecules by proteolytic cleavage. Thus, along with an increase in syndecan ectodomain levels, it is imperative to demonstrate a concomitant decrease in cell surface syndecan levels. When both shed and cell surface syndecan levels are increased, this is most likely due to increased expression and not shedding. This can be confirmed by measuring syndecan mRNA levels. Cell surface syndecan-1 levels can be measured by mild trypsinization and dot immunoblotting of trypsinates. Syndecan-1 ectodomain is apparently cleaved once in the dibasic sequence near the plasma membrane when treated with low levels of trypsin, releasing intact syndecan-1 ectodomains into the culture supernatant. Other syndecans also contain the dibasic sequence, and cell surface levels of mouse syndecan-2 and -3 have been measured by mild trypsinization and dot immunoblotting with polyclonal anti-syndecan-2 and -3 antibodies. However, syndecan-4 ectodomains are extensively hydrolyzed by trypsin and antigenic epitopes for both monoclonal and polyclonal anti-syndecan-4 antibodies are lost even at low doses of trypsin. Thus, flow cytometry is used to measure cell surface syndecan-4 levels (31).

3.1. Measurement of Syndecan Ectodomains by Dot Immunoblotting

1. NMuMG cells are cultured in DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% FBS and 10 µg/mL insulin. It is best to perform agonist-induced syndecan shedding assays with confluent or 1 day post-confluent NMuMG cells to maximize the difference between baseline and induced shedding.

2. Wash confluent or 1-day post-confluent NMuMG cells in 96-well plates once with culture medium. Add agonists diluted in culture medium and incubate for the desired time periods at 37°C. In the example shown in Fig. 1, we incubated NMuMG cells with 1 μ M PMA for 0, 15, or 30 min.
3. At the end of incubation, collect the culture supernatant, transfer to microfuge tubes, centrifuge at $1,000 \times g$ for 15 min to remove detached cells and debris, and transfer the conditioned medium to clean microfuge tubes. Cells remaining on culture vessels are immediately processed for measurement of cell surface syndecan-1 by mild trypsinization and dot immunoblotting as described below in Subheading 3.2.
4. Pre-wet Immobilon Ny+ membranes and 3MM papers in distilled water. Place one sheet of wet 3MM paper on the dot blot apparatus and place one sheet of wet Immobilon Ny+ membrane on top of the 3MM paper. Close the dot blot apparatus and connect to a bench-top vacuum line.
5. Dot blot syndecan standards. In Fig. 1, 0.06, 0.11, 0.16, 0.22, 0.29, and 0.38 ng of purified syndecan-1 ectodomains diluted in 100 μ L of ABS-T were dot blotted onto the Immobilon Ny+ membrane. Dot immunoblotting is a highly sensitive method that can detect as low as 0.05 ng of native syndecan-1 ectodomains in biological fluids.
6. To obtain measurements within the linear range of standards, dot blot 20–80 μ L of the conditioned medium onto Immobilon Ny+ membranes (see Note ⁶). The volume to be blotted depends on the strength of the syndecan shedding agonist and the level of syndecan expression. For example in Fig. 1, we dot blotted 35 μ L of the conditioned medium in triplicate because NMuMG cells abundantly express syndecan-1 and PMA is a strong and rapid inducer of syndecan-1 shedding.
7. Add 1 times volume of ABS-T to each well to acidify samples. Because the isoelectric point (pI) of syndecan ectodomains is considerably lower than pH 4.5 due to the highly anionic HS chains, syndecan ectodomains retain a net negative charge in the ABS-T solution. Further, the pI of syndecan-1 (~4.3), -2 (~4.0), -3 (~4.3), and -4 (~3.8) core proteins is lower than pH 4.5. Thus, by acidifying samples, only highly anionic molecules, such as syndecans, are bound to the cationic Immobilon Ny+ membrane while most proteins pass through the membrane during dot blotting, allowing partial purification of syndecans with this procedure (see Note ⁷).
8. Apply vacuum to the dot blot apparatus and confirm that all samples completely pass through the membrane. With the vacuum running, add 200 μ L of ABS-T to each well and repeat this wash procedure once (see Note ⁸).
9. Turn the vacuum off, disassemble the dot blot apparatus, and remove the membrane. Label the membrane (e.g., experiment number, date, name) (see Note

⁶Air can get trapped in the membrane while applying samples. If the wells appear clogged, turn the vacuum off and tap the side of the dot blot apparatus using caution not to spill the samples in wells. If the wells are still clogged, mix the samples in wells by gentle pipetting using a 20–200 μ L micropipet.

⁷Some samples with high protein concentrations, such as serum and some tissue homogenates, precipitate when acidified. To measure syndecan levels in these samples, dot immunoblotting should be performed with neutral buffer, such as TBS, pH 7.5, instead of ABS-T.

⁸Attach a filtering flask trap between the bench-top vacuum source and dot blot apparatus to prevent clogging the vacuum source. Verify that the material of the filtering flask (glass or polypropylene) can withstand the pressure applied by the vacuum source before use.

⁹). Also, immediately wash the dot blot apparatus thoroughly to eliminate cross contamination in future experiments.

10. Block the membrane with Blocking buffer 1: TBS containing 10% (w/v) nonfat dry milk for 1 h at room temperature with agitation on a bench-top orbital shaker.
11. Discard Blocking buffer 1 and incubate the membrane twice with Blocking buffer 2: TBS with 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 for 30 min at room temperature.
12. Incubate with 0.2 µg/mL of 281–2 antibody diluted in Blocking buffer 2 for 2–4 h at room temperature or overnight at 4°C with agitation on an orbital shaker (see Note ¹⁰).
13. Wash the membrane twice with Blocking buffer 2 for 20 min at room temperature (see Note ¹¹).
14. Incubate with HRP-conjugated secondary antibodies at the dilution recommended by the vendor (1:5,000 for the HRP-conjugated anti-rat antibody in Fig. 1) in Blocking buffer 2 for 2 h at room temperature or overnight at 4°C with agitation on an orbital shaker.
15. Wash the membrane twice with TBS (without milk or Tween-20) for 30 min at room temperature with agitation.
16. Develop membrane with ECL reagent for 1–2 min at room temperature. Remove membrane from ECL solution, remove remnants of ECL solution by blotting with 3MM paper, place membrane in a clear plastic wrap, and press out bubbles from the surface of the membrane.
17. Place the membrane in an image reader and acquire image of the developed blot in 1–5 min intervals. If using film, place the membrane in a film cassette, expose to X-ray films for the desired time periods (e.g., 10 s to 15 min), and develop films with a developer. Scan the acquired image or developed film with commercially available software (e.g., Multi Gauge, Fujifilm) or public domain software (e.g., Image J, NIH Image).
18. Plot the standard graph and quantify dots within the linear range of the standard curve using graph software (e.g., Prism, Kaleidagraph, Excel).

3.2. Measurement of Cell Surface Syndecan-1 by Mild Trypsinization and Dot Immunoblotting

1. Immediately after collecting the conditioned medium, wash NMuMG cells remaining on 96-well plates twice with ice-cold TBS/EDTA.
2. Incubate cells with 10 µg/mL TPCK-treated trypsin in TBS/EDTA for 10 min at 4°C. The volume depends on the culture vessel used. For example, when using 96-well plates, add 50 µL of the TPCK-trypsin solution to each well.
3. Add an equal volume (e.g., 50 µL for 96-well plates) of 100 µg/mL soybean trypsin inhibitor to each well and incubate for 5 min on ice to inactivate trypsin.

⁹We recommend using VWR lab markers (fine tip or extra fine tip) because other markers tend to wash away while immunoblotting.
¹⁰For best results, we recommend incubating blotted membranes in primary and secondary antibodies overnight at 4°C. This reduces background.

¹¹Use caution not to dry the Immobilon Ny+ membrane while immunoblotting. After the membrane is developed, it can be stored dry in a plastic wrap.

4. Transfer trypsinates to microfuge tubes, centrifuge at $1,000 \times g$ for 15 min to remove cells and debris, and transfer trypsinates to clean microfuge tubes.
5. Perform dot immunoblotting as described above in Subheading 3.1, steps 4–18. The volume of trypsinates to be dot blotted depends on the expression level of cell surface syndecans and strength of the shedding agonist. For example in Fig. 1, we dot blotted 35 μL of the conditioned medium in triplicate because NMuMG cells abundantly express syndecan-1 and PMA is a strong inducer of syndecan-1 shedding.

3.3. Measurement of Cell Surface Syndecans by FACS

1. Stimulate confluent or 1 day post-confluent NMuMG cells in 6-well plates with agonist. In the example shown in Fig. 2, NMuMG cells were stimulated with 1 μM PMA for 15 or 30 min at 37°C.
2. Collect culture supernatants and proceed to measurement of shed syndecan ectodomains by dot immunoblotting (see Subheading 3.1 above). Wash remaining NMuMG cells once with PBS.
3. Incubate NMuMG cells with PBS containing 5 mM EDTA (PBS/EDTA) for 30 min at 4°C.
4. Remove cells from each well by scraping on ice and transferring the cell suspension to microfuge tubes.
5. Pellet cells by centrifugation at $1,000 \times g$ for 10 min at 4°C and wash 3 times with ice-cold PBS.
6. Incubate cells with 10 $\mu\text{g}/\text{mL}$ of 281–2 anti-syndecan-1 or 20 $\mu\text{g}/\text{mL}$ of Ky8.2 anti-syndecan-4 antibodies in PBS with 1% (w/v) BSA (BSA/PBS) for 1 h at 4°C. Centrifuge at $1,000 \times g$ for 10 min at 4°C and wash 3 times with ice-cold BSA/PBS.
7. Incubate cells with Alexa 488 donkey anti-rat antibodies for 1 h at 4°C (see Note 12). Wash cells 3 times with ice-cold BSA/PBS.
8. Fix cells with 2% PFA in PBS for 10 min on ice. Wash fixed cells 3 times with ice-cold PBS.
9. Determine the level and number of cells expressing membrane-bound syndecan-1 and -4 by measuring the fluorescence associated with the cell surface at 519 nm using a flow cytometer (see Fig. 2 for examples).

3.4. Stripping and Reprobing Blots for Shed and Cell Surface Syndecans

1. Multiple syndecans can be measured from the same samples by stripping and reprobing blotted membranes with different anti-syndecan antibodies (see Note 13).
2. Wash previously immunoblotted membrane twice with TBS for 30 min at room temperature.

¹²Other fluorophore-conjugated secondary antibodies (e.g., FITC-, PE-, GFP-conjugated antibodies) can also be used. Alternatively, some pre-labeled anti-syndecan antibodies are available commercially (e.g., Cell Sciences, Abcam) and antisyndecan antibodies can be directly labeled using labeling kits available from commercial sources (e.g., Alexa fluor labeling kits from Invitrogen).

¹³When stripping and reprobing, we recommend determining shedding of syndecans that are expressed at lower levels first because the antigenicity of the blotted samples decreases every time the blot is stripped and reprobed. For example, if examining both syndecan-1 and -4 shedding from the same sample, it is better to determine syndecan-4 levels before those of syndecan-1. For the same reason, we do not recommend stripping and reprobing a membrane more than 2 times.

3. Warm the Restore Western Blot Stripping Buffer to room temperature. Add sufficient volume of stripping buffer to cover the whole surface of the membrane.
4. Incubate at 37°C for 30 min with agitation. We either insert the membrane in a hybridization bottle and incubate in a temperature-controlled, rotating hybridization oven or place the membrane in a plastic container and incubate in a temperature-controlled shaking incubator (e.g., bench-top shaker used to grow bacteria).
5. Discard stripping buffer. Wash membrane twice with TBS for 30 min.
6. Start immunoblotting as described above in Subheading 3.1 from step 10.

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Fig. 1. Time-dependent induction of syndecan-1 shedding by PMA measured by dot immunoblotting. Confluent normal murine mammary gland (NMuMG) cells in 96-well plates were incubated with 1 μ M PMA for 0, 15, or 30 min at 37°C in triplicate. For quantification of shed syndecan-1 ectodomains, 35 μ L of the conditioned medium was dot blotted onto the Immobilon Ny+ membrane. For quantification of cell surface syndecan-1, following removal of the conditioned medium, cells were washed once with ice-cold TBS/EDTA and incubated with 50 μ L of ice-cold 10 μ g/mL TPCK-treated trypsin for 10 min at 4°C. Trypsin was inactivated with 50 μ L of 100 μ g/mL soybean trypsin inhibitor. Trypsinates (35 μ L) were dot blotted onto the Immobilon Ny+ membrane. The membrane was immunoblotted with 281-2 antibodies, developed, and quantified as described. (a) Picture of confluent NMuMG cells. (b) Developed dot blot. (c) Standard curve of syndecan-1. (d) Quantification of shed and cell surface syndecan-1 at various times post-PMA stimulation.

Fig. 2. Time-dependent reduction of cell surface syndecan-1 and -4 by PMA measured by FACS. Confluent NMuMG cells in 6-well plates were incubated with 1 μ M PMA for 15 or 30 min at 37°C. Cells were detached by incubating with PBS/EDTA and treated with primary antibodies (281-2 for syndecan-1; Ky8.2 for syndecan-4) and Alexa 488 anti-rat secondary antibodies. After fixing cells with 2% paraformaldehyde (PFA), the fluorescence associated with cell surfaces was measured by FACS. *Black line*, cell surface syndecans without PMA treatment; *light gray line*, cell surface syndecans 15 min after PMA treatment; *dark gray line*, cell surface syndecans 30 min after PMA treatment; and *dotted line*, background fluorescence measured with secondary antibody only.

Table 1

Partial list of syndecan-1 shedding agonists and sheddases

Shedding agonists	Sheddases
Phorbol esters (PMA, TPA) (24)	MMP7 (Matrilysin) (14, 32)
Sphingomyelinase/Ceramide (24)	MMP9 (gelatinase B) (37)
EGF family growth factors (24, 29)	MMP14 (MT1-MMP) (38)
FGF-2 (32)	ADAM17 (TACE) (39)
Insulin (33)	<i>Streptococcus pneumoniae</i> (pneumococcus) ZmpC (23)
TNF α (34, 35)	<i>Bacillus anthracis</i> (anthrax) Npr599 and InhA (40)
RANTES (36)	
SDF-1 (37)	
Cellular stress (hyperosmolarity, heat shock) (24)	
Reactive oxygen species (12)	
Heparanase (10)	
<i>Pseudomonas aeruginosa</i> LasA (27)	
<i>Staphylococcus aureus</i> α - and β -toxin (26)	
<i>Bacillus anthracis</i> AnIB, AnIO, and ClnA (28)	