Heparan Sulfates Mediate the Interaction between Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) and the $\alpha_{q/11}$ Subunits of Heterotrimeric G Proteins*

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Background: The mechanisms by which the PECAM-1-$\alpha_{q/11}$ mechanosensitive complex mediates endothelial flow responses remain unclear.

Results: The PECAM-1-$\alpha_{q/11}$ complex contains heparan sulfate proteoglycans (HSPGs) and is disrupted by inhibition of HS.

Conclusion: The interaction between PECAM-1 and $\alpha_{q/11}$ may be mediated by the HS of the HSPG syndecan-1.

Significance: Targeting specific HSPGs may be an effective strategy for the therapeutic treatment of vascular diseases.

The endothelial cell-cell junction has emerged as a major cell signaling structure that responds to shear stress by eliciting the activation of signaling pathways. Platelet endothelial cell adhesion molecule-1 (PECAM-1) and heterotrimeric G protein subunits $\alpha_q$ and 11 ($\alpha_{q/11}$) are junctional proteins that have been independently proposed as mechanosensors. Our previous findings suggest that they form a mechanosensitive junctional complex that discriminates between different flow profiles. The nature of the PECAM-1-$\alpha_{q/11}$ interaction is still unclear although it is likely an indirect association. Here, we investigated the role of heparan sulfates (HS) in mediating this interaction and in regulating downstream signaling in response to flow. Co-immunoprecipitation studies show that PECAM-1-$\alpha_{q/11}$ binding is dramatically decreased by competitive inhibition with heparin, pharmacological inhibition with the HS antagonist surfen, and enzymatic removal of HS chains with heparinase III treatment as well as by site-directed mutagenesis of basic residues within the extracellular domain of PECAM-1. Using an in situ proximity ligation assay, we show that endogenous PECAM-1-$\alpha_{q/11}$ interactions in endothelial cells are disrupted by both competitive inhibition and HS degradation. Furthermore, we identified the heparan sulfate proteoglycan syndecan-1 in complexes with PECAM-1 that are rapidly decreased in response to flow. Finally, we demonstrate that flow-induced Akt activation is attenuated in endothelial cells in which PECAM-1 was knocked down and reconstituted with a binding mutant. Taken together, our results indicate that the PECAM-1-$\alpha_{q/11}$ mechanosensitive complex contains an endogenous heparan sulfate proteoglycan with HS chains that is critical for junctional complex assembly and regulating the flow response.

The vascular endothelium is constantly exposed to hemodynamic forces (i.e. cyclic stretch, hydrostatic pressure, and fluid shear stress) from blood flow that act on the cells and lead to a variety of cellular responses, including cell morphology, intracellular signaling, and gene expression. With regard to fluid shear stress, these responses can be physiological or pathological depending on the type, magnitude, and direction of flow. Identification of the primary mechanosensor that enables vascular endothelial cells (ECs)² to discriminate between different flow profiles has been a major challenge in the field, although a number of candidate molecules, putative macromolecular complexes, and/or cell structures have been proposed (1, 2).

The endothelial cell-cell junction has been described as the region of highest tension in a continuous EC monolayer under flow (3). At this location, ECs are believed to undergo rapid (within minutes) structural adaptations (i.e. inclination) in response to flow that are followed by activation of downstream signaling (4–6). Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a transmembrane glycoprotein that is abundantly expressed by ECs and primarily localized to cell-cell junctions. In response to fluid shear stress, PECAM-1 is rapidly tyrosine-phosphorylated (30 s), which was concluded to be a result of force application directly to the molecule rather than to the cell (7). Heterotrimeric G proteins are membrane-associated proteins that are activated within seconds of fluid shear stress stimulation (8) and that may be direct (9) or indirect via activation of G protein-coupled receptors (GPCRs) (10). In vivo, the G protein subunits $\alpha_q$ and $\alpha_{11}$ ($\alpha_{q/11}$) are co-localized with PECAM-1 at the cell-cell junction in the straight portions of the mouse descending aorta, an area described as “athero-protected” due to the presence of high, unidirectional laminar flow (11). Interestingly, $\alpha_{q/11}$ is absent from the cell-cell junction at aortic branch points, which are characterized as having large temporal gradients and/or reverse flow and, therefore, “atheroproteone.” $\alpha_{q/11}$ is also absent from the junctions in the descending aorta of the PECAM-1 knockout mouse. In vitro, PECAM-1 and $\alpha_{q/11}$ indeed form a complex, as demonstrated

² The abbreviations used are: EC, endothelial cell; PECAM-1, platelet endothelial cell adhesion molecule-1; GAG, glycosaminoglycan; HSPG, heparan sulfate (HS) proteoglycan; HCAEC, human coronary artery endothelial cell; PLA, proximity ligation assay; BKB2, bradykinin receptor B2; GPCR, G protein-coupled receptor; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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by co-immunoprecipitation experiments using primary human ECs, which is quickly dissociated in response to temporal changes in shear stress (11).

The endothelial glyocalyx layer, a complex structure composed of various proteoglycans, glycosaminoglycans (GAGs), glycoproteins, and plasma proteins that lines the apical surface of endothelial cells, has also been proposed to be the “mechanosensor” that senses fluid shear stress and transmits this force into biological responses in the cell. Evidence for this concept comes from experiments using enzymes that degrade specific glyocalyx components showing that depletion of heparan sulfates abolished shear stress-induced endothelial nitric oxide (NO) production (12) and also prevented endothelial cell alignment in response to flow (13). Among the GAGs associated with the endothelial glyocalyx layer are heparan sulfate (HS), chondroitin sulfate, and hyaluronan/hyaluronic acid. These linear chains of distinct disaccharide unit repeat (a uronic acid and a hexosamine) are generally attached in varying numbers to core proteins and are collectively referred to as proteoglycans. Heparan sulfate proteoglycans (HSPGs) are the most abundant proteoglycans in the vasculature and are typically classified into three subfamilies based on their location: cell surface or membrane-bound (i.e. syndecans and glypicans), secreted extracellular matrix (i.e. perlecan, agrin, collagen XVIII), and secretory vesicle (i.e. serglycin) (14).

A variety of proteins, such as growth factors, cytokines, chemokines, enzymes, enzyme inhibitors, and extracellular matrix proteins, are known to bind to HSPGs (14). It has also been described that an interaction between PECAM-1 and GAGs of the heparin/HS family exists and that the main heparin-binding site for this interaction requires both Ig domains 2 and 3 (15). Coincidentally, we showed that the interaction between PECAM-1 and Gαq/11 was drastically diminished in the absence of Ig domains 2 and 3 of PECAM-1 (16). We, therefore, tested the hypothesis that GAG chains attached to a putative heparan sulfate proteoglycan are part of a mechanosensitive cell-cell junctional complex that contains PECAM-1, Gαq/11, and their respective GPCR(s). We also examined whether their presence as a mediator of physical interactions between components of this macromolecular complex is critical for the flow response.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 cells were obtained from ATCC (Manassas, VA) and maintained in DMEM + GlutaMax-I with d-glucose and sodium pyruvate (Invitrogen) supplemented with 10% heat-inactivated FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin in a humidified 5% CO2 incubator at 37 °C. Human coronary artery endothelial cells (HCAECs) were obtained from Cell Applications, Inc. (San Diego, CA) and maintained in complete endothelial growth medium (EGM-2; Lonza, Walkersville, MD) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin. HCAECs within six passages were used for all experiments.

Reagents—Anti-FLAG mononclonal antibody (M2) was from Sigma. Rabbit monoclonal antibody against Gαq/11 (clone #47) was custom-designed and provided to us by Epitomics (Burlingame, CA). Mouse anti-human heparan sulfate (10E4) was from United States Biological (Swampscott, MA). Anti-phospho-Akt (Ser-473) was from RD Systems (Minneapolis, MN), and anti-Akt was from Santa Cruz Biotechnology (Santa Cruz, CA). Heparin, surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide), and heparinase III (from Flavobacterium heparinum) were purchased from Sigma.

Vector Construction—PECAM-1-FLAG and Gαq expression constructs were produced in pcDNA3.1/hygromycin (pcDNA3.1/Hyg) (Invitrogen) as previously described (16). Site-directed mutagenesis was performed (Genewiz, Inc., South Plainfield, NJ) to generate single, double, triple, and quadruple amino acid FLAG-tagged PECAM-1 mutants. Specifically, lysine was replaced with alanine at position 176 (K176A arginine was replaced with alanine at position 179 (R179A); both the lysine at position 176 and the arginine at position 176 were replaced with alanines (K176A/R179A); lysine at position 176, arginine at position 179, and histidine at position 239 were replaced with alanines (K176A/R179A/H239A); lysine at position 176, arginine at position 179, histidine at position 239, and lysine at position 255 were replaced with alanines (K176A/R179A/H239A/K255A).

Transfection—HEK293 cells were seeded in 6-well plates the day before transfection at a density of 7.5 × 10⁵ cells/well. Transfection of expression constructs were performed using Effectene transfection reagent (Qiagen, Valencia, CA). Cells were collected and lysed 24 h post-transfection. Transfection of FLAG-tagged PECAM-1 constructs into HCAECs was performed using the HCAEC Nucleofector kit with the Amaza Nucleofector II Device (Lonza) following the manufacturer’s optimized protocol and applying Nucleofector Program S-005. Briefly, 5 × 10⁵ cells resuspended in 100 μl of Nucleofector Solution were combined with 2 μg of plasmid DNA for each cuvette. After adding pre-equilibrated medium, cells were transferred to culture dishes and incubated at 37 °C.

siRNA-mediated Knockdown—Knockdown of Gαq/11 expression was performed using a siRNA oligonucleotide designed to target a sequence common to both human Gαq and Gα11 gene transcripts (5'-AAGATGTTCGTGGACCTGAA-3'). Silencing of PECAM-1 was performed using a siRNA oligonucleotide that targets a sequence in the 3'-UTR region of the gene (5'-AAG-GGCCAAGGGCATTGGGATA-3'). Both custom oligonucleotides were obtained from Qiagen. 150 nM final concentration of siRNA were combined to Lipofectamine siRNAmix (Invitrogen) according to the manufacturer’s protocol and added to HCAECs for 4–6 h before cells were reseeded at high density onto glass microscope slides. Cells were then allowed to re-establish confluency (24–48 h) before exposure to shear stress.

Shear Stress—HCAECs were seeded onto glass microscope slides and grown into confluent monolayers. Before all experimental procedures, cells were serum-starved overnight in endothelial basal medium (EBM-2, Lonza) supplemented with 0.5% bovine serum albumin (BSA; Roche Applied Science) and penicillin-streptomycin to establish quiescence. Slides were mounted on a conventional parallel-plate flow chamber (17), and cells were subjected to a steady fluid shear stress of 14 dyne/cm² by perfusion with CO₂-equilibrated EBM-2 containing 0.5% BSA using a PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA). Cells on slides that were
mounted but not subjected to shear stress, denoted “Sham,” served as controls.

Preparation of Cell Lysates and Immunoprecipitation—Cells were scraped into ice-cold Dulbecco’s modified phosphate buffer and collected by centrifugation. Pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 60 mM octyl-glucoside) containing protease inhibitors (Complete; Roche Applied Science). Lysates were incubated for 30 min on ice and then centrifuged at 14,000 g for 15 min at 4 °C to remove insoluble material. Cell lysates were incubated with immobilized anti-FLAG M2 monoclonal antibody-agarose beads and rotated for 2 h at 4 °C. The beads were then washed four times with lysis buffer before elution by boiling in NuPAGE LDS sample buffer (Invitrogen) with XT reducing agent (Bio-Rad).

Western Blot Analysis—Proteins were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) in MOPS SDS running buffer (Invitrogen) and transferred to PVDF membranes (Immobilon-P; Millipore, Temecula, CA). Membranes were blocked for 1 h with 3% BSA in Tris-buffered saline (TBS) and then incubated with a primary antibody for 2 h or overnight in 3% BSA-TBST (TBS with 0.1% Tween 20) at 4 °C. After washing and incubating with horseradish peroxidase-conjugated secondary antibodies for 1 h, the membranes were incubated with chemiluminescence substrate (SuperSignal West Pico or West Femto; Thermo Scientific, Rockford, IL) and exposed to x-ray film. Unsaturated films were digitally scanned, and band intensities were quantified using ImageJ (National Institutes of Health).

Flow Cytometry—HEK293 cells were fixed with 4% paraformaldehyde for 15 min and washed thoroughly with PBS. Cells were then incubated with anti-heparan sulfate IgM for 20 min followed by FITC-conjugated anti-mouse IgM for 20 min with multiple PBS washes in between. Samples were analyzed for HS surface expression using a flow cytometer (FACSCalibur; BD Biosciences).

In Situ Proximity Ligation Assay (PLA)—PLA was performed on HCAEC monolayers that were fixed in ice-cold methanol/acetic acid, rehydrated in PBS, then blocked and treated according to the manufacturer’s protocol (Olink Biosciences, Uppsala, Sweden). Primary antibodies used were goat anti-PECAM-1 (9G11; R&D Systems, Minneapolis, MN), rabbit anti-PECAM-1 (PASS310; ProSci, Poway, CA), rabbit anti-Gαq/11 (clone #47; Epitomics), mouse anti-syndecan-1 (DL-101; Santa Cruz Biotechnology), rabbit anti-syndecan-4 (Santa Cruz Biotechnology), mouse anti-glypican-1 (EMD Millipore, Billerica, MA), goat anti-CXCR4 (Santa Cruz Biotechnology), and mouse anti-CXCR7 (R&D Systems). Images were acquired on a LSM5 PASCAL confocal fluorescence microscope (Carl Zeiss, Germany) equipped with a Plan Apochromatic 63/1.4 numerical aperture oil immersion objective, and both the PLA signal (i.e. single dots or pixels) and cell nuclei were quantified using a custom ImageJ image analysis macro. A minimum of 10 fields of acquisition was acquired for each of the three independent experiments.

Statistical Analysis—All experimental data are expressed as the means ± S.E. from at least three independent experiments. Single comparisons between groups were performed using Student’s t test. p values of <0.05 were considered statistically significant.

RESULTS

Heparin Inhibits Gαq/11 Binding to PECAM-1—To determine whether the interaction between PECAM-1 and Gαq/11 is mediated by HS, we examined the effect of heparin as a competitive inhibitor of HS-protein interactions in co-immunoprecipitation studies. HEK293 cells were co-transfected with plasmid constructs encoding PECAM-1-FLAG and Gαq/11. Lysates were then prepared and subjected to immunoprecipitation of PECAM-1 with an anti-FLAG antibody in the presence or absence of unfractonated heparin. In the absence of heparin, Gαq/11 was detected in anti-FLAG immunoprecipitates (Fig. 1A), which confirmed our previous findings that Gαq/11 is capable of complexing with PECAM-1 (16). Although Gαq/11 was still detected in immunoprecipitates in the presence of heparin, there was a significant decrease (63%; n = 4, p = 0.008) in the level pulled down by PECAM-1 when normalized to the total amount of Gαq/11 available in the cell lysate and expressed as a ratio over immunoprecipitated PECAM-1-FLAG, suggesting that disruption of HS-protein interactions has an inhibitory effect on PECAM-1 association with Gαq/11.

Pharmacological Inhibition of GAGs Blocks PECAM-1-Gαq/11 Association—We also tested the effect of a recently described small molecule antagonist of heparan sulfate, bis-2-methyl-4-aminono-quinolyl-6-carbamide (also known as surfen) (18), on the ability of PECAM-1 to form a complex with Gαq/11. Preincubation of HEK293 cells co-transfected with FLAG-tagged PECAM-1 and Gαq/11 with surfen at 100 μM for 2 h before co-immunoprecipitation resulted in a 62% decrease in Gαq/11 binding to PECAM-1 compared with that found in cells treated with DMSO as a vehicle control (Fig. 1B). This finding further supports the concept that a HS-protein interaction is important for bringing PECAM-1 and Gαq/11 together in a complex. However, because surfen has been reported to also bind dermatan sulfate and chondroitin sulfate, we could not rule out the possibility that other GAGs may be involved in mediating the PECAM-1-Gαq/11 interaction.

PECAM-1-Gαq/11 Association Is Abrogated by Selective Enzymatic Degradation of Heparan Sulfate—We next evaluated whether the reduced binding we observed is due specifically to protein-HS interactions by selectively removing HS by enzymatic digestion. It has been previously shown that HEK293 cells endogenously express HSPGs (19, 20), and therefore, treatment of HEK293 with heparinase III could potentially affect HSPG-protein interactions. Preincubation of HEK293 cells with 200 milliunits of heparinase III for 1 h resulted in a 40% decrease in heparan sulfate cell surface expression as detected by flow cytometry (Fig. 1C). Importantly, this decrease in heparan sulfate expression led to a 59% decrease in co-immunoprecipitation of Gαq/11 with PECAM-1 (Fig. 1C).

Gαq/11 Binding Is Dependent on the Presence of Basic Residues within Ig Domains 2 and 3 of PECAM-1—Molecular modeling studies have previously identified a high affinity binding site located in Ig domains 2 and 3 of PECAM-1 that binds heparin oligosaccharides (21). Specifically, docking simulations indicated that the interaction between a heparin pentasaccharide
and Ig domains 2 and 3 involves seven amino acids: Lys-176, Leu-177, Arg-179, His-239, Lys-255, Gln-259, and Ile-258 (21). We, therefore, used site-directed mutagenesis to generate mutant PECAM-1 constructs targeting the four basic residues contained within this predicted binding site and changing them into alanines. To determine which of the four basic residues was required for the interaction between PECAM-1 and Go<sub>q/11</sub>, we performed co-immunoprecipitation experiments using mutant PECAM-1 constructs that contained a single amino acid substitution (Mut K176A and Mut R179A), a double amino acid
substitution (Mut K176A/R179A), a triple amino acid substitution (Mut K176A/R179A/H239A), or a quadruple amino acid substitution (Mut K176A/R179A/H239A/K255A) (Fig. 1D). As expected, Gαq/11 co-immunoprecipitated with WT PECAM-1 from lysates of co-transfected HEK293 cells. Expression of Mut K176A and Mut R179A led to a modest decrease in co-immunoprecipitation of PECAM-1 and Gαq/11 (17 and 30%, respectively). Expression of Mut K176A/R179A/R197A resulted in a more appreciable decrease in binding of Gαq/11 to PECAM-1 (53%), but there was no statistical difference in the decrease in PECAM-1-Gαq/11 association until at least the third (70%; n = 4, p = 0.0137) and fourth residues (73%; n = 3, p = 0.0288) within Ig domain 3 were mutated. These findings suggest that HS mediates Gαq/11 binding to PECAM-1 through this site.

Endogenous PECAM-1-Gαq/11 Associations Are Mediated by HS—We next investigated the physical associations between PECAM-1 and Gαq/11 in endothelial cells using in situ PLA to validate our in vitro findings in a more biologically relevant cell system. Consistent with our previous findings in vivo in atherosclerotic mouse aortas as well as in vitro in human umbilical vein endothelial cells (11), a junctional complex of endogenous PECAM-1 and Gαq/11 was also observed in situ in HCAECs. To determine whether the PECAM-1-Gαq/11 complex that we observed under basal conditions is dependent on interactions with HS, we tested the effects of competitive binding of heparin and heparinase III treatment. There was a dramatic decrease in the number of PECAM-1-Gαq/11 complexes detected in HCAECs treated with heparin for 5 min (Fig. 2A). Similarly, enzymatic removal of HS with heparinase III for 3 h resulted in a significant decrease in PECAM-1-Gαq/11 association (Fig. 2B). In control experiments, we assessed the association of heparan sulfates directly with Gαq/11, which showed a robust PLA signal that was dramatically decreased with heparinase III treatment (Fig. 2C). We also examined whether increasing the concentration of serum albumin has an effect on PECAM-1-Gαq/11 associations. Our results showed that there was a significant increase upon increasing BSA levels from 0.5% to 1% during overnight starvation (Fig. 2D). Together, these results suggest that the association between PECAM-1 and Gαq/11, within the proposed multimeric complex at the cell-cell junction is mediated through interactions with HS.

PECAM-1 Is Associated with Endogenous HSPGs and GPCRs in Endothelial Cells—Three syndecan family members (syndecans-1, -2, and -4) and one glypican member (glypican-1) of the membrane-bound cell surface HSPGs are known to be expressed by endothelial cells (22, 23). Syndecans-1 and -4, but not syndecan-2, have previously been shown to be in a complex with the chemokine RANTES and its specific GPCRs, CCR1, and CCR5 (24, 25). In situ PLA was, therefore, performed to examine the association of PECAM-1 with these two syndecans and glypican-1 as candidate HSPGs that potentially mediate the interaction between PECAM-1 with Gαq/11 via their respective GPCRs. Our results revealed that all three HSPGs examined are present in complexes with PECAM-1 under basal conditions in HCAECs (Fig. 3, B–D). Interestingly, only syndecan-1 is dissociated from its complex with PECAM-1 in response to flow as indicated by the significant decrease in PLA signal at early time points (7 and 15 s) when compared with that of sham control (Fig. 3B). Glypican-1 had the opposite effect, increasing in its association with PECAM-1 at early flow onset (Fig. 3D). In control experiments, we observed a significant decrease in junctional PECAM-1-Gαq/11 proximity at identical time points (Fig. 3A), which is suggestive of a rapid dissociation upon activation of the complex. Intriguingly, we also observed a dramatic increase in both PECAM-1-syndecan-1 and PECAM-1-syndecan-4 associations at the 60-s time point. To verify that the associations between PECAM-1 and syndecan-1 are localized to the cell-cell junction of ECs, we counterstained cells with a FITC-conjugated antibody against PECAM-1 after performing PLA. Co-localization of the PECAM-1-syndecan-1 complex with junctional PECAM-1 was clearly observed in quiescent HCAECs (Fig. 4A). Because the GPCR CXCR4 is known to form a complex with syndecan-4, we examined its possible association with PECAM-1. As shown in Fig. 4B, CXCR4 is indeed associated with PECAM-1 in quiescent ECs but is not localized specifically to the junction. We also examined the association between PECAM-1 and CXCR7, another GPCR in the chemokine receptor family that signals through the same ligand as CXCR4. Surprisingly, PECAM-1 and CXCR7 displayed strong co-localization at the EC junction under basal conditions (Fig. 4C).

Heparan Sulfate Removal Attenuates Flow-induced Akt Phosphorylation—Because heparinase III treatment resulted in a decreased association between PECAM-1 and Gαq/11, as shown by co-immunoprecipitation in HEK293 cells and by in situ PLA in HCAECs, we proposed that the flow response in these cells would also be compromised. To test our hypothesis, we examined the phosphorylation of Akt as a marker of down-
Disruption of heparin/heparan sulfates affects association between endogenous PECAM-1 and \( \alpha_{q/11} \). A, in situ PLA was performed using antibodies directed against PECAM-1 and \( \alpha_{q/11} \) on HCAECs that were left untreated or treated with heparin (500 \( \mu \)g/ml) for 5 min. Representative confocal images of cells are shown. Red dots indicate close proximity of PECAM-1 and \( \alpha_{q/11} \), at the cell-cell junction. Blue staining (DAPI) indicates nuclei. Quantification is depicted in the bar graph as PLA signal per cell relative to the untreated condition with error bars indicating S.E. of three independent experiments. \( n = 3 \); ***,

\( p < 0.001 \).

B, PLA performed on HCAECs that were left untreated or treated with 100 milliunits heparinase III for 2 h. \( n = 3 \); ***,

\( p < 0.001 \).

C, PLA performed using antibodies directed against heparan sulfate and \( \alpha_{q/11} \) on HCAECs that were left untreated or treated with 100 milliunits of heparinase III for 2 h. \( n = 3 \); ***,

\( p < 0.001 \).

D, PLA was performed using antibodies directed against PECAM-1 and \( \alpha_{q/11} \) on HCAECs that were serum-starved in the presence of 0.5% BSA (control) or 1% BSA. \( n = 4 \); ***,

\( p < 0.001 \).
FIGURE 3. Endogenous HSPGs are associated with PECAM-1. In situ PLA was performed using antibodies directed against PECAM-1 and Gαq/11 (A), PECAM-1 and syndecan-1 (SDC1) (B), PECAM-1 and syndecan-4 (SDC4) (C), or PECAM-1 and glypican-1 (GPC1) (D) on HCAECs that were unstimulated (Sham) or exposed to flow for the indicated times. Representative confocal images of cells with the indicated conditions are shown. Each bar graph shows quantification of three independent experiments as PLA signal (red dots) per cell (blue nuclei) relative to the sham condition with error bars indicating S.E. n = 3; *, p < 0.05; ***, p < 0.001.

FIGURE 4. Syndecan-1 and CXCR7 are co-localized with PECAM-1 at the cell-cell junction. In situ PLA was performed using antibodies directed against PECAM-1 and SDC1 (A), PECAM-1 and CXCR4 (B), or PECAM-1 and CXCR7 (C) on quiescent HCAECs. Cells were counterstained with FITC-conjugated anti-PECAM-1 to identify the cell-cell junctions. Representative confocal images from three independent experiments are shown.
stream signaling in response to flow. Our results indicate that in HCAECs, in which heparan sulfate was enzymatically removed with heparinase III before flow exposure, Akt phosphorylation was significantly decreased when compared with untreated control cells (Fig. 5A).

**Flow-induced Akt Activation Is Decreased in Cells That Express Heparin-binding Mutant PECAM-1—**To determine whether there are any functional consequences to the specific disruption of the PECAM-1-Ga_q/11 interaction, we performed reconstitution experiments in which endogenous PECAM-1 was knocked down and replaced by either wild type or heparin-binding mutant PECAM-1 (Fig. 5B). siRNA targeting a sequence in the 3′-UTR of PECAM-1 mRNA was transfected into cells, which resulted in an ~90% decrease in the expression of PECAM-1 as assessed by Western blotting. Akt phosphorylation was markedly decreased in HCAECs in which PECAM-1 protein was knocked down before flow exposure ($n = 4; p < 0.05$; $**$, $p < 0.01$ versus siControl flow condition).
or the quadruple amino acid substituted PECAM-1 (Mut K176A/R179A/H239A/K255A) on Akt phosphorylation in PECAM-1-silenced HCAECs. Cells that were reconstituted with WT PECAM-1 to a level greater than 2-fold over cells transfected with PECAM-1 siRNA alone did not show a significant flow-induced decrease in Akt phosphorylation compared with siControl-transfected cells under flow conditions. On the contrary, flow-induced Akt phosphorylation remained significantly decreased in cells reconstituted with a similar level of mutant PECAM-1 (n = 4; p = 0.0285).

**DISCUSSION**

Endothelial mechanotransduction is a normal physiological process by which the endothelium converts forces from blood flow into biochemical responses, but these same forces can also cause pathological responses leading to EC dysfunction and atherogenesis. Paramount to understanding how ECs sense and respond to distinct flow patterns is the identification of the primary mechanosensor. Although PECAM-1 and Gq/11 have been independently proposed as primary mechanosensors, our previous studies established that these two junctional proteins form a complex that is mechanosensitive and rapidly responds to temporal gradients in shear stress but not to steady fluid flow (11, 16). The exact mechanism by which PECAM-1 and Gq/11 act together to sense and respond to early flow onset remains to be elucidated. The objective of this study was to elucidate the aspects of the immediate mechanotransduction pathway of transient shear stress, which has been shown to lead to an atherogenic phenotype both in vitro and in vivo (26–28). To this end, we investigated the role of HS in mediating the interaction between PECAM-1 and Gq/11 using an in vitro model system to simulate the atherogenic component of physiological flow and examined the effect that disruption of this interaction may have on shear stress-induced signaling.

HEK293 cells are one of the most widely used cell lines for heterologous expression of proteins because they allow for high efficiency of transfection in addition to high fidelity of translation and processing of proteins (29). For these reasons and because these cells express high mRNA levels of numerous GPCRs (30), this expression system was utilized to determine the nature of PECAM-1-Gq/11 interactions. We found that HS are critically involved in PECAM-1-Gq/11 association within the basal complex based on the observations that heparin, surfen, and heparinase III treatments all caused a marked decrease in their binding when co-expressed in HEK293 cells. We also examined endogenous interactions in situ using PLA in endothelial cells to validate our findings and to rule out the possibility of overexpression artifact. The strengths of this particular approach compared with traditional biochemical methods, such as co-immunoprecipitation, are that 1) it enables detection of protein interactions as they naturally occur in the cell, and 2) even weak and transient interactions are detectable.

Although the enzymatic removal of heparan sulfates that extend from the apical surface of endothelial cells by treatment with heparinase III has been a widely used approach for determining the role of the endothelial glyocalyx layer as a primary mechanosensor, its potential effects on protein interactions mediated by HSPGs have been generally overlooked in those studies. PECAM-1 represents one such protein that has been shown to bind to HS (15, 31) and whose function, particularly as a critical mediator of the flow response, may be significantly altered by removal of HS. Therefore, studies using heparin and surfen were performed to complement those in which HS was enzymatically digested by heparinase III. Heparin, at a concentration range of 50–500 μg/ml, has previously been shown to inhibit PECAM-1-mediated aggregation of mouse L cell fibroblasts (32). It has also been reported that heparin at low concentrations (100 μg/ml) blocks lipoprotein binding to HSPGs (33), but higher concentrations (5–10 mg/ml) are necessary for releasing receptor-mediated binding of lipoproteins to human fibroblasts (34). In our studies, heparin at an intermediate concentration (500 μg/ml) was used to competitively bind to PECAM-1 and prevent its binding to HS. Indeed, the presence of heparin resulted in both decreased binding of Gq/11 to PECAM-1 when co-expressed in HEK293 and decreased association of the endogenous proteins in HCAECs. Surfen, on the other hand, was used based on its ability to bind to HS. It was utilized in this manner and shown to inhibit HS-mediated signaling of FGF2 and VEGF through their respective receptors (18, 35). In the case of exogenous addition of either heparin or surfen, the endothelial glyocalyx layer remains fully intact so any downstream signaling effect would be indicative of disruption of protein-GAG interactions rather than to the loss of a reputed primary mechanosensor.

Site-specific mutations were introduced into the Ig domains 2 and 3 of PECAM-1 based on a report that predicted a cluster of basic amino acids located 20 Å apart as being critical for the interaction between PECAM-1 and heparin/HS (21). The significance of this distance is that other known heparin-binding proteins, including type IV collagen, neural cell adhesion molecule (N-CAM) and apolipoprotein E, all have a similar spatial distribution of basic amino acids that accommodates a GAG pentasaccharide regardless of whether their three-dimensional structure consists of α-helices or β-strands (36). Another important point is that the targeted amino acids in our studies here are distinct from those identified in Ig domain 1 that are required for mediating homophilic binding of PECAM-1 (37). By knocking down endogenous PECAM-1 in cells and reconstituting them with PECAM-1 that contains these heparin binding mutations, we are specifically targeting heterophilic interactions between PECAM-1 and HS-containing proteins without affecting homophilic interactions between PECAM-1 molecules that are known to be crucial for cell-cell junctional formation (38, 39). We found that this cluster of four basic amino acids was important not only for the interaction between PECAM-1 and Gq/11, but also for the activation of Akt in response to flow. Our results regarding flow-induced Akt phosphorylation in the absence of PECAM-1 using an siRNA targeting approach is similar to those previously published using human umbilical vein endothelial cells exposed to longer periods of shear stress (40). However, our study differs substantially from the other in that we show experimentally that the decrease in Akt phosphorylation is due specifically to the lack of PECAM-1 function rather than to the lack of PECAM-1 protein as reconstitution of the cells with WT PECAM-1, but not mutant PECAM-1, leads to a renewed ability of cells to respond...
to flow changes. The decrease in flow-induced Akt phosphorylation is also quite similar to that we previously reported in human umbilical vein endothelial cells when Goq/11 is silenced by siRNA (6). The incomplete restoration of Akt phosphorylation upon reconstitution of PECAM-1-silenced cells with WT PECAM-1 could be attributed to the lower levels of PECAM-1 expression compared with that present in control siRNA-transfected HCAECs (~70% less) and/or to the relatively low transfection efficiency (up to 57%) of DNA plasmids into HCAECs by the nucleofection method. In other words, the majority of cells lack PECAM-1 due to the high efficiency of siRNA transfection, but less than half the cell population contains the WT PECAM-1 expression construct. The observed elevation in Akt phosphorylation by mutant PECAM-1-reconstituted cells compared with PECAM-1-silenced cells, albeit not significant, may be due to the inability of the quadruple amino acid substitution to completely disrupt the assembly of the putative mechanosensitive complex. Together, these results support the concept that the association of Goq/11 with PECAM-1 at the cell-cell junction is critical for the flow response, particularly Akt signaling.

It was previously shown that in response to a sudden temporal onset of flow (1-s impulse), Goq/11 is rapidly dissociated from PECAM-1 (15 and 30 s) followed by re-association at later time points (60 and 180 s) as demonstrated by co-immunoprecipitation in human umbilical vein endothelial cells (11). In rapid response to a step change in shear stress, we observed a similar pattern of decreased association between Goq/11 and PECAM-1 at early time points (7 and 15 s) in HCAECs by PLA. It is worth noting that we observed an increase in the association of the PECAM-1-Goq/11 complex in cells starved in the presence of 1% BSA as opposed to 0.5% BSA, which supports the notion that serum albumin concentrations <1% may lead to a partial collapse of the glycocalyx (41) and provides further evidence that HS mediates the interaction between the two proteins.

Using the same PLA methodology, we found that PECAM-1 is closely associated with three different HSPGs: syndecan-1, syndecan-4, and glypican-1. Although syndecan-4 remained closely associated with PECAM-1 in response to step flow, syndecan-1 rapidly dissociated (7 and 15 s) and re-associated (30 and 60 s). This pattern of dissociation and re-association is strikingly similar to that we observed for PECAM-1 and Goq/11 suggesting that syndecan-1 is part of the same mechanosensitive complex with PECAM-1 and Goq/11. The observation that both syndecan-1 and syndecan-4 interactions with PECAM-1 are increased at 60 s is consistent with flow-induced junctional clustering of glycocalyx components (i.e., HS and glypican-1), which has been recently reported to occur in rat fat pad endothelial cells at 30 min (42). In the case of HCAECs there seems to be a more rapid association of syndecans with the junction. In contrast, association of PECAM-1 with glypican-1 increases with early flow onset and does not appear to be localized to the cell-cell junction. Interestingly, both PECAM-1 and glypican-1 have been independently shown to be expressed in caveolin-1-containing membrane fractions (43, 44), with the latter believed to be involved in shear-induced NO production through a glypican-caveola-endothelial nitric-oxide synthase mechanism (45). Additionally, it has been recently reported that PECAM-1 and caveolin-1 form a mechanosensing complex that is necessary for NAPDH oxide 2 (NOX2) and angiogenic signaling in pulmonary endothelial cells in response to abrupt cessation of flow (44). Therefore, it is not inconceivable that a subset of PECAM-1 forms a functionally distinct mechanosensitive caveolar complex together with glypican-1.

There is increasing evidence indicating that syndecan-1 may serve as a bridge in bringing GPCR/Goq/11, to the endothelial cell-cell junction. First, syndecan-1 has previously been shown to interact with GPCRs, including CCR1 and CCR5 (24, 25). Secondly, syndecan-1 has been reported to be expressed strongly along cell-cell junctions of epithelial cells (46). Finally, syndecan-1 was observed to be co-localized with junctional PECAM-1 in HCAECs in the present study. Furthermore, because the HS chains on syndecan-1 and syndecan-4 are structurally indistinguishable and essentially identical with regard to their ligand binding affinities (46), it is possible that syndecan-1, as opposed to syndecan-4, is the specific HSPG utilized by endothelial cells.

It was previously demonstrated that the GPCR bradykinin receptor B2 (BKR2) interacts with PECAM-1 and enhances the association between PECAM-1-Goq/11 when co-transfected in HEK293 cells (16). Intriguingly, a direct interaction between BKR2 and PECAM-1 was not established in those studies. A possible explanation for this is that BKR2 interacts indirectly with PECAM-1 through direct associations with HSPGs. Other endogenous GPCRs besides BKR2 are likely involved, as the PECAM-1-Goq/11 interaction can be detected without co-expressing BKR2, and GPCRs are well known to form heterodimers and even hetero-oligomers (47, 48). It is interesting to note that the chemokine receptor CXCR4, which has previously been shown to interact with syndecan-4 (49), is highly expressed in HEK293 cells (30). However, HEK293 also express mRNAs of at least 75 other GPCRs (30) for which endogenous interactions with HSPGs have not yet been described and, therefore, cannot be excluded from consideration.

It has recently been reported that the chemokine receptor CXCR7 is co-localized with PECAM-1 in tumor endothelial cells of bladder tissue, and its overexpression causes increased Akt phosphorylation in the bladder carcinoma cell line HT1376 (50). Our results here extend those findings in that we show that association of CXCR7 with PECAM-1 occurs primarily at the cell-cell junctions in HCAECs. Additionally, CXCR4 and CXCR7 are known to form heterodimers, which have been shown to enhance CXCL12-induced signaling (51, 52). Therefore, CXCR7 either alone or together with CXCR4 may be the putative GPCRs that are directly bound to syndecans, which in turn are bound to junctional PECAM-1. Our results from studies in HEK293 cells and HCAECs suggest a model in which Goq/11 is coupled to hetero-oligomerized GPCRs that are localized at the junction through HS-mediated interactions with PECAM-1. These GPCRs may include BKR2, CXCR7, and CXCR4, the latter of which has been shown to be coupled to Goq/11 contrary to the notion that it only signals through Goq/11 activation (53). Future studies directed at identifying which these endogenous GPCR(s) in endothelial cells interacts specifically with syndecan-1 should shed more light not only on the
composition of the junctional mechanosensitive complex but also on the mechanism by which temporal changes in flow leads to activation of downstream signaling pathways.

Collectively, our data suggest that a mechanosensitive complex containing PECAM-1, GPCR/Gαq/11, and HSPG(s), presumably syndecan-1, resides at the endothelial cell-cell junction during quiescence and is primed for activation upon sensing temporal changes in shear stress. Interactions between the proteins in this complex appear to be mediated by HS as its targeted disruption leads to dissociation of Gαq/11 from PECAM-1 and to an abrogated flow response. To our knowledge this is the first study to demonstrate interplay between the junctional complex containing PECAM-1 and Gαq/11 and components of the glycocalyx, providing a unifying model for endothelial mechanosensing in response to temporal changes in shear stress. In light of our findings, uncoupling of PECAM-1 from Gαq/11 at the EC cell-cell junction through heparin mimetics or specific targeting of HSPGs such as syndecan-1 may represent a therapeutic approach for treating flow-induced vascular diseases.

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