Mechanotransduction in an extracted cell model: Fyn drives stretch- and flow-elicited PECAM-1 phosphorylation

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Mechanosensing followed by mechanoresponses by cells is well established, but the mechanisms by which mechanical force is converted into biochemical events are poorly understood. Vascular endothelial cells (ECs) exhibit flow- and stretch-dependent responses and are widely used as a model for studying mechanotransduction in mammalian cells. Platelet EC adhesion molecule 1 (PECAM-1) is tyrosine phosphorylated when ECs are exposed to flow or when PECAM-1 is directly pulled, suggesting that it is a mechanochemical converter. We show that PECAM-1 phosphorylation occurs when detergent-extracted EC monolayers are stretched, indicating that this phosphorylation is mechanically triggered and does not require the intact plasma membrane and soluble cytoplasmic components. Using kinase inhibitors and small interfering RNAs, we identify Fyn as the PECAM-1 kinase associated with the model. We further show that stretch- and flow-induced PECAM-1 phosphorylation in intact ECs is abolished when Fyn expression is down-regulated. We suggest that PECAM-1 and Fyn are essential components of a PECAM-1–based mechanosensory complex in ECs.

Introduction

Mechanical force regulates a variety of physiological processes involved in cellular functions, development of tissues and organs, and the health of an organism (Orr et al., 2006). Although the mechanisms by which cells sense and convert mechanical force into intracellular biochemical signals have been a subject of keen interest, the problem is not easy to approach experimentally. However, some experimental systems have been developed in recent years to study specific mechanotransduction pathways. For example, Sheetz and his associates made detergent-extracted cell models and in vitro protein extension systems that can elicit certain mechanoresponses and be analyzed biochemically (Sawada and Sheetz, 2002; Tamada et al., 2004; Sawada et al., 2006). We, and others, have attached microbeads coated with either antibodies or ligands specific for cell surface proteins onto cultured cells and mechanically stimulated the cells by tugging on the beads (Osawa et al., 2002; Tzima et al., 2005; Wang et al., 2005). These studies are beginning to reveal molecular mechanisms for mechanotransduction by specific proteins.

Endothelial cells (ECs) are known to respond to fluid shear stress and mechanical stretch and are regarded as one of the best known mammalian cell systems for studying mechanotransduction. Ion channels, integrins, glycocalyx, and G protein–coupled receptors are thought to be involved in fluid shear stress sensing by ECs because either flow provokes their activities or disruption of their function down-regulates certain flow-dependent responses (Davies, 1995; Resnick et al., 2003; Li et al., 2005). However, it remains largely unknown how these molecules convert mechanical force into intracellular signaling. Platelet EC adhesion molecule 1 (PECAM-1) is a cell adhesion molecule localized to interendothelial contacts. It forms trans-homophilic associations extracellularly and contributes to the formation and maintenance of an EC monolayer. In addition to being a cell adhesion molecule, it appears to have roles in cell signaling, as its short cytoplasmic domain contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), a motif known to be

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Abbreviations used in this paper: BAEC, bovine aortic EC; EC, endothelial cell; ERK, extracellular signal-regulated kinase; HAEC, human arterial EC; HUVEC, human umbilical vein EC; ITIM, immunoreceptor tyrosine-based inhibitory motifs; PECAM-1, platelet EC adhesion molecule 1; VEGFR, VEGF receptor.

The online version of this paper contains supplemental material.

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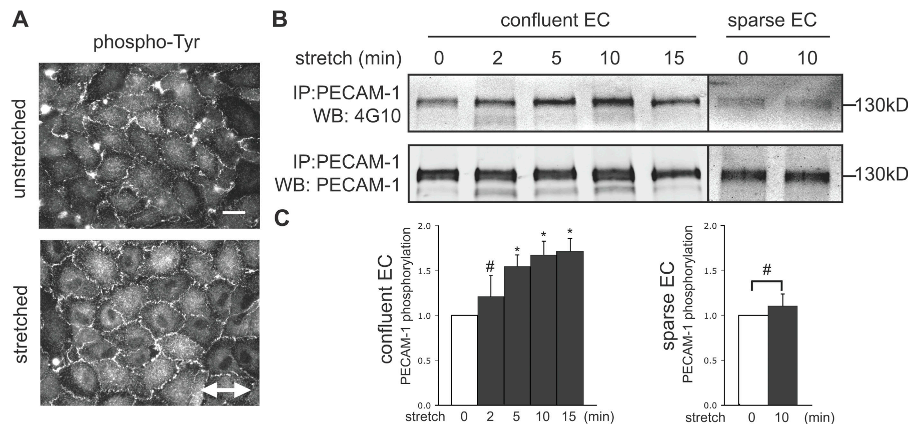

Figure 1. **Stretch-induced PECAM-1 phosphorylation in intact ECs.** (A) Confluent BAECs cultured on an elastic substrate were stretched (25% elongation) for 5 min or left unstretched and stained with anti–protein phosphotyrosine (4G10). Tyrosine phosphorylated proteins were associated with interendothelial contacts, but this localization pattern became more prominent in stretched cells. Bipolar arrow indicates the direction of stretch. Bar, 20 µm. (B) Confluent or sparse BAECs cultured on an elastic substrate were subjected to stretch (25% elongation) for the times indicated or left unstretched. PECAM-1 phosphorylation was analyzed by immunoprecipitating PECAM-1 and immunoblotting with anti–PECAM-1 and 4G10. Although PECAM-1 phosphorylation increased in confluent cells, no increase was observed in sparse cultures. Black lines indicate that intervening lanes have been spliced out. (C) Quantification of immunoblotting results. Relative levels of PECAM-1 phosphorylation were determined by measuring the intensity of immunoblotted bands (see Materials and methods) and expressed relative to the phospho–PECAM-1 level in unstretched cells (mean ± SEM). Sample size: $n = 6$ for confluent samples, $n = 3$ for sparse samples. Student's *t* test was used to compare each time point with unstretched sample for each category. *, P = 0.0007, 0.0006, and 0.0003 for 5, 10, and 15 min, respectively. #, P = 0.0733 (confluent, stretched 2 min) and 0.5077 (sparse, stretched 10 min).

involved in signaling (Woodfin et al., 2007). When the tyrosine residue in the ITIM is phosphorylated, it associates with SHP-2 (SH2 domain–containing protein tyrosine phosphatase) and activates the extracellular signal-regulated kinase (ERK) signaling pathway (Milarski and Saltiel, 1994; Jackson et al., 1997). We have found that when cultured ECs are exposed to physiological levels of shear stress, PECAM-1 ITIMs are phosphorylated and that this phosphorylation mediates ERK activation by fluid shear stress (Masuda et al., 1997; Osawa et al., 2002; Tai et al., 2005). Interestingly, these shear stress–dependent responses occur when a pulling force is applied directly to PECAM-1 on the cell surface using magnetic beads coated with antibodies against the external domain of PECAM-1 (Osawa et al., 2002). This experiment suggests that PECAM-1 responds directly to mechanical force, transducing mechanical force into a biochemical signal. Because PECAM-1 has no intrinsic kinase activity, some kinase must be involved in PECAM-1 phosphorylation, and identifying the kinase is essential to elucidating the mechanism for PECAM-1 mechanotransduction.

The presence of a mechanotransducer protein complex at cell–cell contacts has been previously suggested (Davies et al., 2003; Chiu et al., 2004; Liebner et al., 2006), and PECAM-1 may be a key molecule in such a complex (Osawa et al., 2002; Bagi et al., 2005; Tzima et al., 2005). We hypothesized that the kinase that phosphorylates PECAM-1 might be a component of such a complex. In this study, we made a detergent-extracted cytoskeletal model of EC monolayers that retained PECAM-1 localization at interendothelial contacts and investigated PECAM-1 tyrosine phosphorylation in it by stretch. We found that when the EC model was stretched in the presence of ATP, PECAM-1 was phosphorylated. Because PECAM-1 phosphorylation did not require exogenous kinase, the kinase that phosphorylated PECAM-1 must be a component of the cell model. Using several inhibitors targeted to different sets of tyrosine kinases, we identified Src, Yes, and Fyn as possible PECAM-1 kinases. Then, we used a siRNA technique to show that Fyn, not Src and Yes, was the kinase required for PECAM-1 phosphorylation in stretched cell models. To investigate if Fyn was also involved in stretch-induced PECAM-1 phosphorylation in intact ECs, we stretched ECs treated with Fyn, Src, or Yes siRNA and found that PECAM-1 phosphorylation was inhibited only by Fyn siRNA. PECAM-1 phosphorylation by flow was also inhibited by Fyn siRNA. Identification of Fyn as the kinase for mechanical force-elicited PECAM-1 phosphorylation provides significant insights into PECAM-1–dependent mechanosignaling in ECs.

Results

Stretch-induced PECAM-1 tyrosine phosphorylation in live and detergent-extracted ECs

Confluent ECs were cultured on an elastic silicone membrane that was uniaxially extended by 5–25% of the original length, causing the cells to be stretched. Increased protein tyrosine phosphorylation, a hallmark of signaling activity, became detectable within a few minutes of stretch, peaked at 10 min, and then gradually diminished (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200801062/DC1). When ECs were stained with anti–protein phosphotyrosine (4G10), interendothelial contacts of stretched cells became more prominently stained (Fig. 1 A), suggesting that cell–cell contacts are a major stretch-induced mechanosignaling site. Initially, experiments were done using cyclic stretch, but this creates movement of the culture medium, causing cells to be exposed to both fluid shear stress and stretch. To avoid this situation, we used a single sustained stretch where the silicon membrane remained stretched throughout the experiment. The membrane was stretched gently so that the shear effect would be small. For example, we took 3 s to stretch the chamber by 7.5 mm (25% stretch), thus exposing cells to a flow velocity of 2.5 mm/s at the beginning of stretch experiments. Although data from cyclic stretch experiments were more variable, cells responded to both forms of stretch in a similar manner (Fig. S2). The data illustrated are from sustained stretch experiments unless otherwise stated.

PECAM-1 is localized to interendothelial cell–cell contacts (Fig. 2 C). It is constitutively tyrosine phosphorylated at low levels, but when confluent ECs are stimulated by flow or when PECAM-1 on the surface of sparsely cultured ECs is directly pulled, PECAM-1 tyrosine phosphorylation increases (Osawa et al., 2002). Stretch is another form of mechanical stress and may also elicit PECAM-1 phosphorylation. Indeed, when bovine aortic ECs (BAECs) were stretched, PECAM-1 phosphorylation increased in confluent but not sparse cultures (Fig. 1, B and C), indicating that this PECAM-1 mechanoresponse is cell–cell contact–dependent or, in molecular terms, only PECAM-1 that is externally engaged can respond to stretch. There was no statistical difference in phosphorylation levels at 5 min and after 5 min, indicating that the plateau is reached by 5 min. This PECAM-1 phosphorylation time course is similar to those by flow and hyperosmotic shock (Osawa et al., 1997). To show that this response was not limited to BAECs, we stretched human umbilical vein ECs (HUVECs) and human arterial ECs (HAECs) and observed that PECAM-1 in these cells also responded similarly (see Figs. 4 D and 7 A). If PECAM-1 phosphorylation is indeed a mechanical event, it might occur when an extracted cell model is stretched.

Confluent BAECs on a silicone substrate were extracted with Tris-buffered saline containing 0.1% Triton X-100 and stretched in the presence of ATP (Fig. 2 A). By scanning electron microscopy, we observed exposed nuclei and a filamentous meshwork in extracted cells (Fig. 2 B). Roughly 70% of the cell protein was extracted, but ~80% of PECAM-1 was retained in cell models of BAECs, HUVECs, and HAECs (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801062/DC1). When models were stained with anti–PECAM-1, a honeycomb pattern similar to that exhibited by unextracted ECs was observed (Fig. 2 C). By immunofluorescence, VE-cadherin and β-catenin localization along the cell border and actin filaments of the circumferential ring appeared unaffected by the extraction (unpublished data).

When EC models were stretched for up to 10 min in the presence of 0.5 mM ATP, 4G10 immunostaining along cell–cell contacts increased (Fig. 3 A) and immunoblots with 4G10 revealed a prominent 130-kD band (Fig. 3 B). When cell models of BAECs whose PECAM-1 expression was down-regulated by siRNA were stretched, phosphorylation of this band was attenuated (Fig. 3 B), suggesting that the protein is PECAM-1. To specifically analyze PECAM-1 phosphorylation in the model, PECAM-1 was immunoprecipitated and its phosphorylation assayed by 4G10 immunoblotting. Both sustained (Fig. 4 A) and cyclic (Fig. 4 C) stretch augmented PECAM-1 phosphorylation in the BAEC model. Cell models of HUVECs and HAECs responded similarly to stretch (Fig. 4 D; and see Fig. 6 B). Stretching models without ATP did not appreciably phosphorylate PECAM-1 (Fig. 4 A). To see if the plateau level of stretch-induced PECAM-1 phosphorylation depended on the magnitude of stretch, models were stretched by 5 (lowest stretch obtainable by the apparatus), 10, 15, 20, and 25% (Fig. 4 B) for 5 min. We found that 5% stretch was as effective as 25% stretch in phosphorylating PECAM-1. Because the stretch apparatus used is not designed to reliably stretch cells <5%, no analysis was done on cells with <5% stretch. Our studies appear to suggest that PECAM-1 phosphorylation is triggered by a simple mechanical process achievable by a 5% extension in length. Our results together show that, other than ATP, soluble components of the cell do not play a significant role in stretch-induced PECAM-1 phosphorylation and that all proteins needed for this stretch response are in the model, presumably as a stable complex. Furthermore, they suggest that PECAM-1 phosphorylation by stretch is a mechanical phenomenon that does not require living cells. We hypothesized that the fundamental mechanism for mechanostimulus-dependent PECAM-1 phosphorylation was preserved in the model and that the kinase for this response was associated with the model.

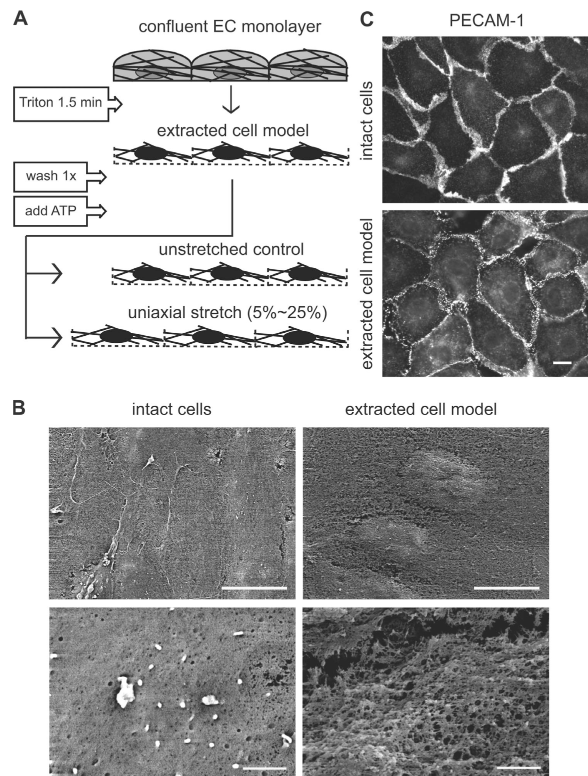

Figure 2. **The detergent-extracted EC model.** (A) Schematic representation of the preparation and mechanical activation of the cell model. Confluent BAECs cultured on an elastic substrate were extracted briefly with Triton X-100. The material remaining attached was considered "the extracted cell model". The model was subsequently incubated with buffer containing ATP and subjected to uniaxial stretch or left unstretched at 37°C. (B) Scanning electron micrographs showing the surface structure of intact cells and extracted cell models. Note that intracellular structures are exposed in extracted cells (right), whereas smooth plasma membrane is seen in intact ECs (left). Bars: (top) 10 µm; (bottom) 1 µm. (C) Immunofluorescence localization of PECAM-1 in intact and extracted BAECs. Cell border localization of PECAM-1 is preserved in the model. Bar, 10 µm.

From 244 known kinases to 3 candidates

Roughly a dozen kinases have been reported to phosphorylate PECAM-1 (Lu et al., 1997; Masuda et al., 1997; Cao et al., 1998; Cicmil et al., 2000; Newman et al., 2001; Ohmori et al., 2001; Kogata et al., 2003) based on in vitro kinase assays and experiments involving inhibitors, overexpression studies, and co-immunoprecipitation, which may or may not reflect what happens

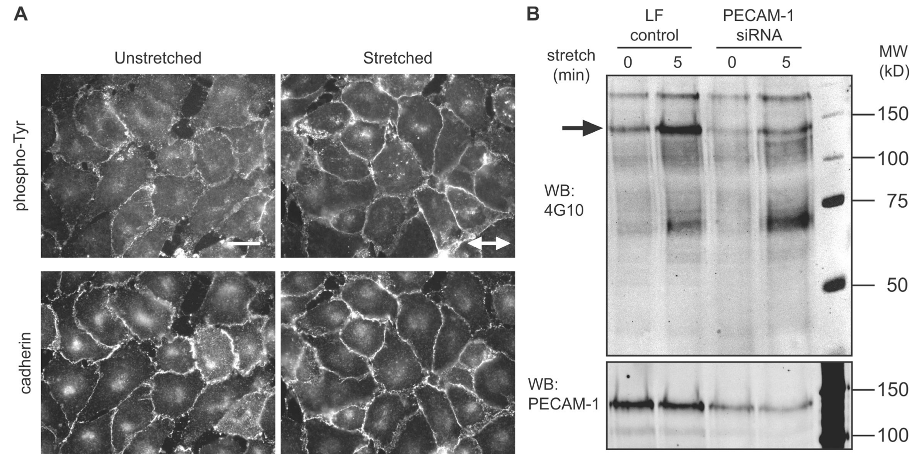

Figure 3. **Increased tyrosine phosphorylation in the stretched cell model.** (A) Extracted BAEC models were stretched or unstretched for 10 min and double labeled with 4G10 and anti–pan-cadherin. Note that cells in the stretched monolayer are more clearly outlined by 4G10 staining than those in the unstretched sample. No significant changes were observed in anti-cadherin staining before and after stretching. Bipolar arrow indicates the direction of stretch. Bar, 20 µm. (B) BAECs cultured on an elastic substrate were treated with Lipofectamine only (LF control) or transiently transfected with PECAM-1 siRNA and then extracted. The extracted models were stretched or left unstretched for 5 min at 37°C and their tyrosine phosphorylated protein levels determined using 4G10 immunoblotting. Arrow indicates a 130-kD band whose phosphorylation level was diminished in PECAM-1 siRNA transfected cells.

inside ECs under physiological conditions. It is possible that PECAM-1 is a promiscuous substrate, but it is also possible that kinases are task specific. Our model may provide a unique opportunity to identify a kinase for a specific PECAM-1 phosphorylation system. Cell models were pretreated for 1 min with a kinase inhibitor, stretched or left unstretched for 5 min in the presence of the inhibitor, Na_3VO_4 (a tyrosine phosphatase inhibitor) and ATP, and then PECAM-1 phosphorylation levels were assayed. For controls, models were stretched without inhibitors, with Na_3VO_4, or with Na_3VO_4 and DMSO (solvent for inhibitors), all of which exhibited stretch-induced PECAM-1 phosphorylation. As shown in Fig. 5 (A and B), 100 µM genistein, 10 µM PP2, 1 µM SU6656, and 1 µM staurosporin (not depicted) but not 10 µM imatinib, 200 µM LFM-A13, and 1 µM VEGF receptor (VEGFR) kinase inhibitor inhibited PECAM-1 phosphorylation by stretch.

Of all the inhibitors we used that blocked PECAM-1 phosphorylation by stretch, the most complete efficacy data were available for PP2. Its effects on 244 known kinases (which also include 14 mutant kinase forms) were obtained from the following database: http://www.invitrogen.com/downloads/SelectScreen_Data_193.pdf. Using this database, we identified 46 kinases whose activity was inhibited by 60% or more by 10 µM PP2 (Table S1, available at http://www.jcb.org/cgi/content/full/

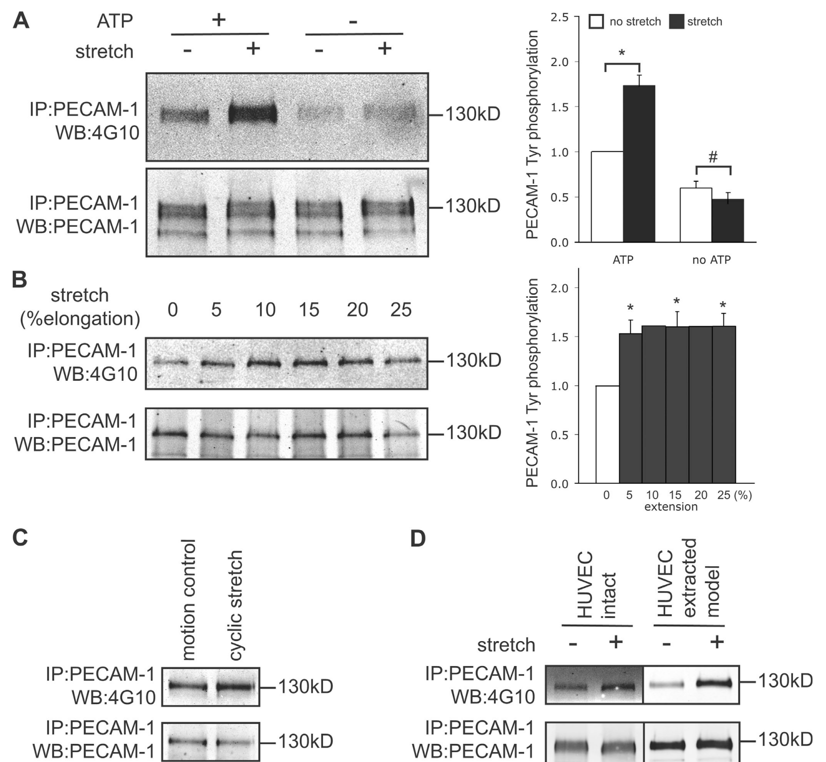

Figure 4. **Stretch-induced PECAM-1 phosphorylation in the cell model.** (A) BAEC models were stretched or left unstretched for 5 min in the presence or absence of ATP. PECAM-1 was immunoprecipitated from solubilized models and immunoblotted with 4G10 and anti–PECAM-1. An example of typical immunoblotting data is shown (left). Intensity of immunoblotted bands was quantified and expressed relative to the PECAM-1 phosphorylation level in ATP-treated unstretched samples (right; mean ± SEM; *n* = 4). Student's *t* test was used to compare stretched to unstretched samples for each category. *, P = 0.0003; #, P = 0.1248. (B) BAEC models were stretched for 5 min to various extents (% elongation) and immunoprecipitated PECAM-1 was immunoblotted with 4G10 and anti–PECAM-1. Levels of phosphorylation were quantified and expressed relative to the PECAM-1 phosphorylation level in unstretched cells (mean ± SEM). Sample size: *n* = 3 for 0, 5, 15, and 25%; *n* = 2 for 10 and 20%. Student's *t* test was used to compare stretched to unstretched samples. *, P = 0.0095, 0.0095, and 0.0049 for 5, 15, and 25%, respectively. (C) Cyclic stretch (15%; 1 Hz; 5 min) induced a 1.53 ± 0.05-fold increase (mean ± SEM; *n* = 3) of PECAM-1 phosphorylation in extracted BAEC models. Control chambers were left on the movable shaft of the stretch apparatus without stretching so that they were exposed to the same oscillation (motion control). Student's *t* test was used to compare stretched to unstretched samples. P = 0.0002. (D) Stretch-induced PECAM-1 phosphorylation in intact and extracted HUVEC. Student's *t* test was used to compare stretched to unstretched samples. In intact cells, stretch induced a 1.42 ± 0.18-fold increase (mean ± SEM; *n* = 5; P = 0.0273). In extracted models, stretch induced a 1.75 ± 0.33-fold increase (mean ± SEM; *n* = 5; P = 0.0260). Black lines indicate that intervening lanes have been spliced out.

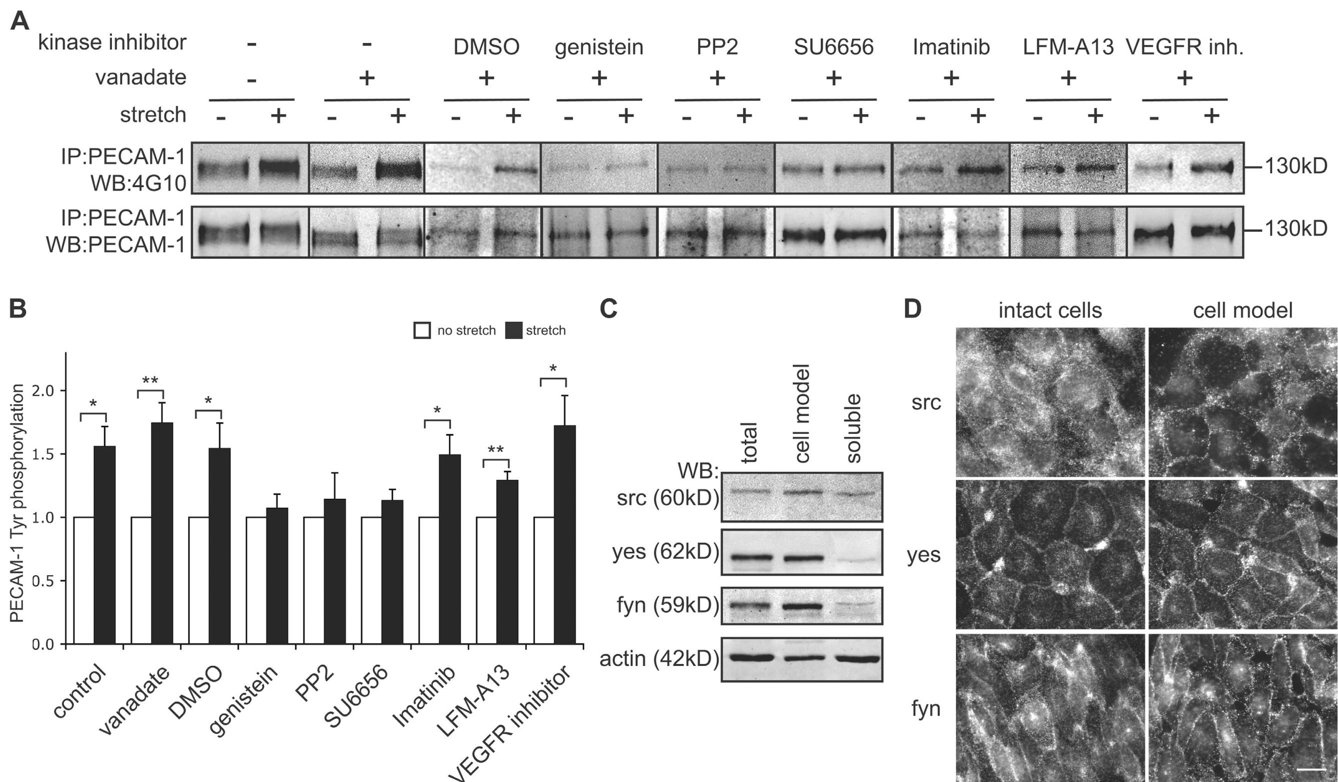

Figure 5. **Kinase inhibitor screening.** (A) BAEC models were pretreated with inhibitors for 1 min at 37°C and stretched (25% elongation) or left unstretched for 5 min in the presence of inhibitors, Na_3VO_4, and ATP. PECAM-1 was immunoprecipitated from solubilized models and immunoblotted with anti–PECAM-1 and 4G10. Note that some inhibitors inhibited PECAM-1 phosphorylation, whereas others did not. Control models were treated with no inhibitor and either Na_3VO_4 or DMSO and Na_3VO_4. Black lines indicate that intervening lanes have been spliced out. (B) Quantification of inhibitor effects on PECAM-1 phosphorylation. Levels of PECAM-1 phosphorylation relative to unstretched models are shown (mean ± SEM; *n* = 3). Student's *t* test was used to compare stretched to unstretched samples for each inhibitor. P = 0.0153 (control), 0.0050 (Na_3VO_4), 0.0117 (DMSO), 0.2800 (genistein), 0.2622 (PP2), 0.0958 (SU6656), 0.0158 (Imatinib), 0.0085 (LFM-A13), and 0.0202 (VEGFR inhibitor). *, $P < 0.05$; **, $P < 0.01$. (C) BAEC whole cell lysates (total), cell models, and detergent-soluble fractions (soluble) were immunoblotted with anti-Src, anti-Yes, or anti-Fyn. Comparable amounts of total protein were loaded in each lane as shown by immunoblots for actin. (D) Src, Fyn, and Yes were localized to interendothelial contacts in both intact cells and cell models. HAECs were used for Src localization, whereas BAECs were used for Yes and Fyn localization. Bar, 20 µm.

jcb.200801062/DC1). From this list, we eliminated 29 based on their lack of endothelial or blood vessel expression, their lack of membrane localization, or their target amino acid specificity (i.e., serine or threonine kinase). Table I shows 17 PP2-sensitive tyrosine kinases that could be a PECAM-1 kinase. The table also includes data on the effects of other inhibitors on each kinase. Because SU6656 inhibited PECAM-1 phosphorylation, any kinase that is not significantly inhibited by this compound cannot be a PECAM-1 kinase. In contrast, because imatinib, LFM-A13, and VEGFR kinase inhibitor did not inhibit PECAM-1 phosphorylation, kinases that are inhibited by these compounds should be eliminated. Out of the 17 remaining kinases, our kinase inhibitor screening eliminated all but Fyn, Src, and Yes as possible PECAM-1 kinases.

We next investigated if these three were present in cell models and localized to cell–cell contacts. Because Src, Fyn, and Yes are members of the Src family kinase and share high sequence homology, we used monoclonal antibodies made against the unique region of each kinase. Src, Yes, and Fyn were detected in cell models and localized to the cell border in both extracted and unextracted ECs (Fig. 5, C and D). Thus, these kinases are associated with cell–cell contacts in the model, and the association is not an artifact of cell extraction.

Fyn, not Src or Yes, is responsible for PECAM-1 phosphorylation in stretched models

Because simple association-based screening could not reduce the field of kinase candidates further, we turned to a molecular biology approach. ECs were treated with Src, Yes, or Fyn siRNA, cell models were made and stretched, and PECAM-1 phosphorylation was assayed. The mean knockdown efficiency estimated by immunoblotting was 60, 67, and 70% for Src, Yes, and Fyn, respectively. For controls, cells were treated with commercially available control siRNA, Lipofectamine treatment alone, or two scrambled Fyn siRNAs and assayed for PECAM-1 phosphorylation by stretch and for expressions of PECAM-1, Src, Yes, Fyn, and Fer (Fig. 6 A). None of these treatments negatively affected the results of these assays. Because the lot and concentration of the Lipofectamine used were the most critical factors for cell viability, monolayer morphology, and transfection efficiency, siRNA results are shown with Lipofectamine controls. No significant inhibition in PECAM-1 phosphorylation was noted in the stretched models of Src (Fig. 6, B and C) or Yes (Fig. 6, D and F) siRNA-treated cells. These results could mean that residual levels of Src and Yes are sufficient for the stretch-induced PECAM-1 phosphorylation but, as we will

Table I. **Kinase inhibitor screening**

Kinases	Inhibitors					
	100 µM Genistein	10 µM PP2	1 µM SU6656	10 µM Imatinib	200 µM LFM-A13	1 µM VEGFR kinase inhibitor
ABL	+	+	−	+		
ABL1 E255K		+		+		
ABL1 G250E		+		+		
ABL1 Y253F		+		+		
ABL2 (ARG)		+		+		
BMX		+		−	+	
CSK		+	−	−		
EGFR	+	+	−	−	−	
ERBB4 (HER4)		+		+		
FGR		+		+		
FLT4 (VEGFR3)		+		−		+
FYN	+	+	+	−		
KDR (VEGFR2)		+		−		+
PDGFRA D842V		+		+		
PDGFRB		+	−	+		+
SRC	+	+	+	−		−
YES	+	+	+	−		

Listed are tyrosine kinases that are PP2 sensitive, expressed in arterial ECs, and likely localized to cell–cell contacts. Their susceptibility to other inhibitors is indicated. −, inhibition by <60% ; +, inhibition by >60%; blank, no data available. PECAM-1 response was inhibited by Genistein, PP2, and SU6656 and was not inhibited by Imatinib, LFM-A13, and VEGFR kinase inhibitor (Fig. 5, A and B). Kinase susceptibility is obtained from the following sources: Genistein, Akiyama and Ogawara (1991); PP2 and Imatinib, http://www.invitrogen.com/downloads/SelectScreen_Data_193.pdf; SU6656, Blake et al. (2000); LFM-A13, Chau et al. (2002) and Mahajan et al. (1999); and VEGFR kinase inhibitor, Cools et al. (2004) and Fraley et al. (2002).

discuss in subsequent sections, this is unlikely. When we tested models of Fyn siRNA-treated cells, the PECAM-1 response was inhibited (Fig. 6, E and F). Because Fyn siRNA-treated cells had normal expression of Src and Yes (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200801062/DC1), if either of them is responsible for the stretch-induced PECAM-1 phosphorylation, the Fyn knockdown should not have inhibited the PECAM-1 response. Thus, the Fyn siRNA experiments argue against the possibility that residual Src and Yes are capable of phosphorylating PECAM-1 in the Src and Yes siRNA-treated cell models. These siRNA experiments collectively indicate that Fyn, not Src and Yes, is responsible for phosphorylating PECAM-1 in stretched cell models.

Fyn phosphorylates PECAM-1 in stretch- and flow-stimulated ECs

To test if Fyn also functions as the sole PECAM-1 kinase in stretched intact cells, the siRNA-treated cells were stretched without extraction. ECs with reduced Src or Yes expression failed to inhibit stretch-induced PECAM-1 phosphorylation (Fig. 7, A–C and E). However, Fyn siRNA again disrupted the PECAM-1 stretch response (Fig. 7, D and E). We used two different Fyn siRNA constructs, each targeting a different region of Fyn mRNA, and obtained similar inhibitory effects, whereas scrambled Fyn siRNA sequences did not reduce Fyn expression nor inhibit PECAM-1 response (Fig. 6 A). Furthermore, Fyn knockdown also inhibited flow-induced PECAM-1 phosphorylation (Fig. 7, F and G). Collectively, our study strongly suggests that Fyn is the kinase for stretch- and flow-induced PECAM-1 phosphorylation.

Although Fyn can directly phosphorylate PECAM-1, it is possible that Fyn's role is to activate Fer (Kapus et al., 2000), which then phosphorylates PECAM-1. This possibility deserves special attention because PECAM-1 was phosphorylated in cells overexpressing Fer (Kogata et al., 2003), even though Fer was eliminated by the first level of inhibitor screening. When BAECs were treated with Fer siRNA and stretched, PECAM-1 phosphorylation was unattenuated, showing that Fer plays no role in this PECAM-1 response (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200801062/DC1). The result also shows that our inhibitor screening has correctly eliminated Fer, providing validity to our approach. When Fyn was knocked down by siRNA, PECAM-1 phosphorylation by stretch and flow was almost completely inhibited. This suggests that other kinases, including undiscovered ones, do not play a major role in PECAM-1 phosphorylation by mechanical stresses. Thus, although other kinases may phosphorylate PECAM-1 under different circumstances, we conclude that Fyn is the kinase responsible for stretch- and flow-induced phosphorylation of PECAM-1.

When ECs are stretched or exposed to flow, Fyn could be activated. To test this possibility, we immunoprecipitated Fyn from stretch- and flow-stimulated ECs and performed immunoblotting on the precipitates using an anti–active Src antibody, which is known to recognize active Fyn. As a positive control, we treated ECs with hyperosmotic shock, which is known to activate Src family kinases (Cohen, 2005), and confirmed highly increased phosphorylation of Src family kinases by immunoblotting with this antibody (unpublished data). However, increased phosphorylation at the active site of Fyn was not observed in ECs exposed to stretch or flow for 10 min (Fig. 7 H). These results are consistent with those reported earlier by Sawada et al. (Sawada et al., 2006). Although these results support the idea

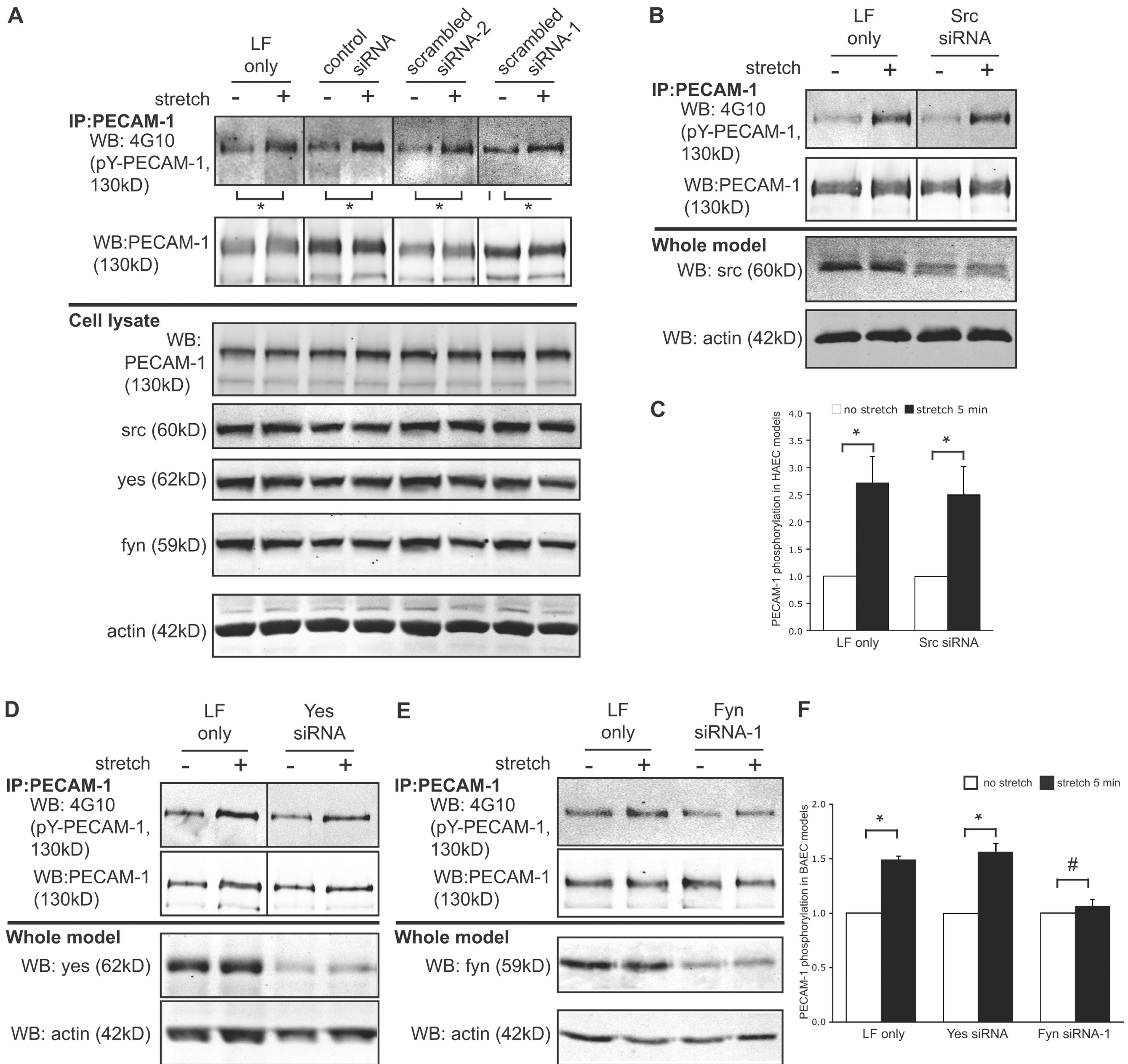

Figure 6. **Fyn is essential for the PECAM-1 mechanoresponse in cell models.** (A) Controls for siRNA experiments. ECs cultured on an elastic substrate were transiently transfected with Lipofectamine 2000 alone, negative control siRNA, or scrambled siRNAs, stretched in the presence of Na_3VO_4 for 10 min, and PECAM-1 tyrosine phosphorylation was assayed (top). Compared with unstretched ECs under each condition, stretched cells exhibited increased PECAM-1 phosphorylation by 1.42 ± 0.08- (LF only; *n* = 6), 1.84 ± 0.15- (control siRNA; *n* = 4), 1.69 ± 0.17- (scrambled siRNA-2; *n* = 3), and 1.49 ± 0.09- (scrambled siRNA-1; *n* = 3) fold (mean ± SEM). Student's *t* test was used to compare stretched to unstretched values. *, P = 0.0002 (LF control), 0.001 (control siRNA), 0.047 (scrambled siRNA-2), and 0.004 (scrambled siRNA-1). The bottom shows the expression of PECAM-1, Src, Yes, and Fyn in whole cell lysates by immunoblotting using actin as a loading control. Although not depicted here, Fer expression was not altered by these control siRNAs. (B–F) ECs cultured on an elastic substrate were transiently transfected with Src, Yes, or Fyn siRNA or Lipofectamine 2000 alone, and cell models were made after they formed confluent monolayers. Models were stretched or left unstretched for 5 min at 37°C in the presence of ATP and Na_3VO_4, and PECAM-1 phosphorylation was assayed. Each kinase associated with the cell model was checked by immunoblotting, using the level of actin as a loading control. (B and C) PECAM-1 phosphorylation was not suppressed in HAEC models with reduced Src expression. PECAM-1 phosphorylation levels are shown relative to unstretched models (mean ± SEM; *n* = 3). Student's *t* test was used to compare stretched to unstretched values. *, P = 0.0124 (LF control) and 0.0292 (Src siRNA). (D) PECAM-1 phosphorylation was not suppressed in BAEC models with reduced Yes expression. (E) Suppressed PECAM-1 phosphorylation in BAEC models with reduced Fyn expression is shown. (F) Levels of PECAM-1 phosphorylation in Yes and Fyn siRNA experiments are quantified and shown relative to unstretched models (mean ± SEM; *n* = 5). Student's *t* test was used to compare stretched to unstretched values. *, P = 1.7×10^{-7} (LF control) and 0.0032 (Yes siRNA); #, P = 0.1665. Black lines indicate that intervening lanes have been spliced out.

that mechanical stress does not activate Fyn, it remains possible that a small fraction of Fyn that is critical for PECAM-1 phosphorylation is activated and that our assay is unable to detect this. Although this possibility cannot be excluded, our results show that Fyn is constitutively active at a significant level in unstimulated cells, suggesting that the early phase of the

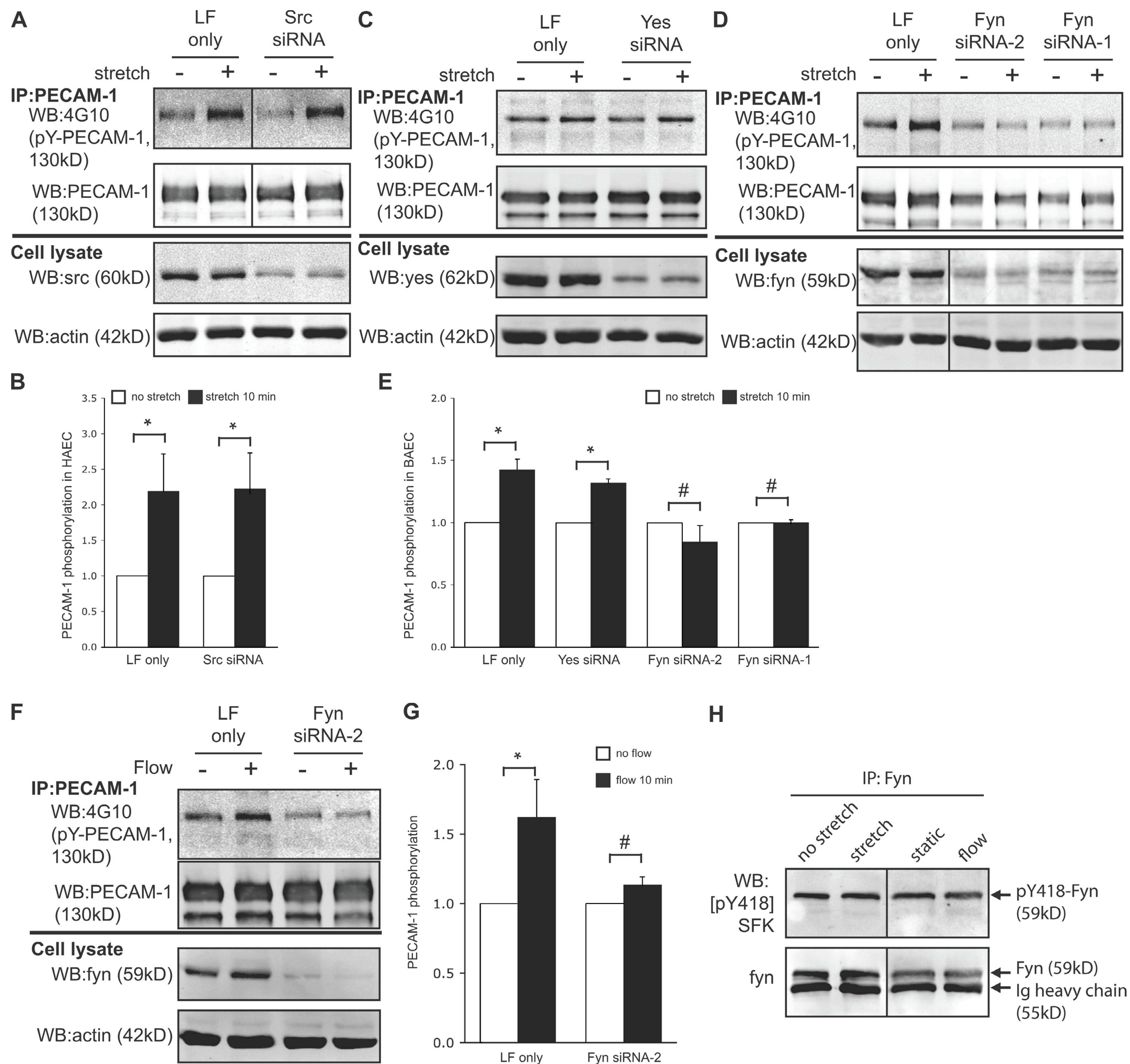

Figure 7. **Fyn mediates stretch- and flow-elicited PECAM-1 phosphorylation in ECs.** ECs cultured on an elastic substrate were transiently transfected with Src, Yes, or Fyn siRNA or Lipofectamine 2000 alone. When confluent monolayers were formed, cells were stretched in the presence of Na_3VO_4 for 10 min and PECAM-1 tyrosine phosphorylation was assayed. Expression of each kinase was checked in whole cell lysates by immunoblotting using actin as a loading control. (A) PECAM-1 phosphorylation was not suppressed in HAECs transfected with Src siRNA. (B) Levels of PECAM-1 phosphorylation in Src siRNA-treated HAECs are quantified and shown relative to unstretched cells (mean ± SEM; *n* = 4). Student's *t* test was used to compare stretched to unstretched values. *, P = 0.0315 (LF control) and 0.0264 (Src siRNA). (C) Reduced expression of Yes in BAECs did not suppress PECAM-1 phosphorylation. (D) PECAM-1 phosphorylation was suppressed in BAECs transfected with two different Fyn siRNAs. (E) Levels of PECAM-1 phosphorylation in Yes and Fyn siRNAs treated cells are quantified and shown relative to unstretched cells (mean ± SEM). The sample sizes are *n* = 4 for Lipofectamine control and Yes siRNA, *n* = 3 for Fyn siRNA-2, and *n* = 5 for Fyn siRNA-1. Student's *t* test was used to compare stretched to unstretched values for each category. *, P = 0.0002 (Lipofectamine control) and 9.8×10^{-7} (Yes siRNA). #, P = 0.1493 (Fyn siRNA-2) and 0.4875 (Fyn siRNA-1). (F) Confluent BAECs transiently transfected with Fyn siRNA or Lipofectamine 2000 alone were subjected to flow (24 dyn/cm^2) in the presence of Na_3VO_4 for 10 min at 37°C, and PECAM-1 phosphorylation was assayed. Flow-induced PECAM-1 phosphorylation was suppressed in Fyn siRNA-treated cells. (G) Quantified results of flow experiments were expressed as the mean ± SEM (*n* = 4). Student's *t* test was used to compare shear stressed to static control values. *, P = 0.0311; #, P = 0.0532. (H) Fyn activity was analyzed in BAECs subjected to stretch or flow for 10 min by immunoprecipitation with anti-Fyn and immunoblot with antibodies that recognize the active form of Src family kinases (pY418 SFK). Note that mechanical stress does not increase Fyn activity. Note also that Fyn is active in unstimulated (unstretched and static) cells. One of three independent experimental results is depicted. Black lines indicate that intervening lanes have been spliced out.

mechanostress-induced PECAM-1 phosphorylation by Fyn is equally possible without robustly activating this kinase.

Discussion

PECAM-1 and mechanotransduction

Our study has demonstrated that PECAM-1 can be phosphorylated when detergent-extracted EC monolayer models are stretched in the presence of ATP, indicating that the model contains molecules that are needed for this stretch-activated phosphorylation reaction. What is not known is whether or not the complete phosphorylation machinery is preserved in the model. For example, it is possible that this in vitro system has lost certain regulatory mechanism and, thus, the model is unregulated. However, it is reasonable to assume that proteins that are minimally needed for PECAM-1 phosphorylation by stretch are in

the model. It is also reasonable to assume that the stretch-induced PECAM-1 phosphorylation reaction occurs without the soluble components of the cell and the plasmalemmal function that regulates ionic events across the membrane, although they could still play roles in fine-tuning the reaction. Using this model system, we identified Fyn as the kinase for stretch-induced PECAM-1 phosphorylation, and using siRNAs, we further demonstrated that Fyn was the primary kinase responsible for PECAM-1 phosphorylation in intact ECs exposed to stretch as well as shear stress.

It is not yet clear how Fyn and PECAM-1 interact in a mechanostress-dependent manner. The fact that PECAM-1 phosphorylation is achievable in the model appears to suggest Fyn and PECAM-1 to be closely positioned, and the two molecules interact when mechanical forces are applied. This could involve certain structural changes, as we had suggested in our earlier study (Osawa et al. 2002). Another possibility is that Fyn located near the PECAM-1 phosphorylation sites is activated when the model is stretched. Highly localized activation of Fyn that our assay cannot detect remains as a possibility, but our study has shown that stretch and flow do not significantly elevate Fyn activity in ECs within the time scale of our experiments and that a portion of Fyn appears to be constitutively active. Thus, Fyn activation may not be necessary. A third possibility is that mechanostress causes already active Fyn to diffuse a small distance to phosphorylate PECAM-1. This diffusion-dependent phosphorylation may be slow and inefficient so that it may take some time for the assay system to detect an increase in PECAM-1 phosphorylation. It is of course also plausible that any combinations of these possibilities occur simultaneously. At present, the precise molecular mechanism for the mechanostress-induced Fyn–PECAM-1 interaction remains elusive.

The presence of a supramolecular complex that converts mechanical force into biochemical reactions at cell–cell contacts has been proposed (Osawa et al., 2002; Davies et al., 2003; Chiu et al., 2004; Tzima et al., 2005; Liebner et al., 2006), but its molecular makeup remains largely unknown. Our earlier experiments, in which PECAM-1 phosphorylation was achieved by a direct application of pulling force onto PECAM-1 which then caused ERK activation, suggest that PECAM-1 converts the mechanical force into biochemical energy (i.e., phosphorylated form of PECAM-1) which then activates intracellular signaling (Osawa et al., 2002). In this regard, PECAM-1 may be a mechanotransducer of the hypothesized mechanoresponsive protein complex. The present study suggests that Fyn as the PECAM-1 kinase is an important component of the mechanotransducer complex. Indeed, small but detectable amounts of Fyn were coimmunoprecipitated with PECAM-1 from platelets (Cicmil et al., 2000; Ohmori et al., 2001), and we also identified a small amount of Fyn in anti–PECAM-1 immunoprecipitates from EC extracts (unpublished data). We wondered if the association between Fyn and PECAM-1 might increase in ECs exposed to stretch, but we were unable to reliably quantify the level of Fyn–PECAM-1 coimmunoprecipitation because the amount of Fyn coimmunoprecipitated with PECAM-1 at any time point during stretch stimulation was barely above the limit of detection (unpublished data). Nonetheless, our past and present studies suggest that PECAM-1 and Fyn are important molecules in a mechanotransducer complex located at cell–cell contacts.

There are mechanosignaling events that depend on PECAM-1 expression such as Src phosphorylation and integrin activation by fluid shear stress (Tzima et al., 2005). This PECAM-1–dependent Src phosphorylation was reported to reach the maximum level within 15 s of flow stimulation, which is much sooner than an increase in PECAM-1 phosphorylation can be reliably detected. If the Src phosphorylation by flow requires PECAM-1 phosphorylation, it might be achieved by the fraction of PECAM-1 that is constitutively phosphorylated. Another possibility is that when the first molecules of PECAM-1 are phosphorylated by flow, which should take place immediately after flow stimulation, a mechanism for Src phosphorylation is fully activated, resulting in the robust activation of Src. A more interesting possibility, however, is that Src phosphorylation does not depend on PECAM-1 phosphorylation. This hypothesis suggests PECAM-1 to be involved in two different mechanosignaling pathways, one PECAM-1 phosphorylation-dependent and one PECAM-1 phosphorylation-independent pathway.

Mechanical response of in vitro models

The use of a detergent-extracted cell model is an important aspect of this study. Not only did it simplify the system but it also allowed us to use various inhibitors effectively. When an inhibitor is used with intact cells, one always wonders what the concentration of the inhibitor is inside them. This ambiguity is practically absent when a cell model is exposed to inhibitors. In addition, because the efficacy data available from the database is based on in vitro assays where inhibitors have free access to target kinases, it is important for our system to be studied with inhibitors under the same barrier-free condition. Several recent studies on mechanotransduction have successfully used cell models to elucidate certain molecular mechanisms for mechanically induced protein–protein interactions and protein phosphorylation (Sawada and Sheetz, 2002; Tamada et al., 2004; Sawada et al., 2006). Our study has added a new dimension, inhibitor screening combined with the siRNA technique, to the use of cell models in the study of mechanotransduction.

Kinase screening

In this study, we used kinase inhibitors to make a short list of possible PECAM-1 kinases and then siRNA to identify Fyn as the PECAM-1 kinase. The same general approach may be applicable to other studies in which a specific kinase or enzyme is to be identified. For example, stretch-induced tyrosine phosphorylation of proteins associated with focal adhesion in detergent-extracted fibroblast models was reported (Tamada et al., 2004), but the study did not identify specific kinases for the response. This would be an excellent system to which the present strategy could be applied. Kinases are particularly good targets because a sufficiently large database is available for kinase inhibitors. For effective screening, it is essential to select a set of inhibitors, some of which do and others of which do not inhibit a particular biological process under investigation. With an appropriate combination of positive and negative inhibitors, one can come up with a short list of possible kinases, which then may be

screened further by other means. Due to the possible inhibitor concentration differential across the plasma membrane, this approach is more applicable to cell-free systems than living cells. A caveat is that the strategy can only be applied to known kinases. Unknown or novel kinases or those for which little to no data are available in the database cannot be studied by this approach. However, this problem should become less of an issue as the information in inhibitor databases expands.

Materials and methods

Reagents

For culturing cells, DME, penicillin/streptomycin, and Trypsin/EDTA solution were purchased from Mediatech, Inc., medium 200 and low serum growth supplement from Cascade Biologics, and FBS from Equitech-Bio, Inc. Other reagents purchased include Lipofectamine 2000 (Invitrogen), collagen type I (BD Biosciences), genistein (EMD), LFM-A13 (EMD), SU6656 (EMD), VEGFR kinase inhibitor (3-(3-Thienyl)-6-(4-methoxyphenyl)pyrazolopyrimidine; EMD), and PP2 (Tocris Bioscience). Imatinib was kindly provided by Novartis. Rabbit polyclonal antibodies against human PECAM-1 (C-20), Fyn (clone Fyn3), and Fer (H-180) were obtained from Santa Cruz Biotechnology, Inc., rabbit anti-Src[pY418] from Invitrogen, and rabbit anti–pan-cadherin from Invitrogen. The following mouse monoclonal antibodies were purchased: anti–protein phosphotyrosine (clone 4G10) and anti-Src (clone GD11; Millipore); anti-c-Yes and anti-Fyn (BD Biosciences); and anti-actin (clone AC-40; Sigma-Aldrich). Fluorescently labeled secondary antibodies used include IRDye 800–conjugated anti–mouse IgG (Rockland), IRDye 680–conjugated anti–rabbit IgG (Rockland), Alexa Flour 488–labeled goat anti–mouse IgG (Invitrogen), and Alexa Flour 546–labeled goat anti–rabbit IgG (Invitrogen). Antibodies against bovine PECAM-1 cytoplasmic domain were made and described previously (Osawa et al., 2002).

Cells and mechanical stimulation

BAECs were purchased from Clonetics, cultured in DME (1 g/liter glucose) supplemented with 10% FBS and penicillin/streptomycin (50 IU/ml and 50 µg/ml) at 37°C and 5% CO_2, and used between passages six and nine. HAECs were purchased from Cascade Biologics and used between passages two and four. HUVECs were kindly provided by Z.-G. Jin and C. Wong (Aab Cardiovascular Research Institute, University of Rochester, Rochester, NY) and used between passages three and eight. Both HAECs and HUVECs were cultured in Medium 200 supplemented with 5% FBS, 2% low serum growth supplement, and penicillin/streptomycin (50 IU/ml and 50 µg/ml) at 37°C and 5% CO_2.

The stretch apparatus was described previously (Shi et al., 2007) and operated in either a cyclic or single sustained stretch mode. Silicone chambers were coated by adding and then evaporating 2 ml of 100 µg/ml Type I collagen in 0.02 N acetic acid at room temperature overnight, UV sterilized, and rinsed five times with PBS. ECs ($2–3 \times 10^5$ cells) were plated on the collagen-coated surface (30×30 mm^2) and cultured for 2–3 d to reach confluence. To stimulate ECs with shear stress, cells were grown to confluency in a gelatin-coated 60-cm Petri dish for 2–3 d and exposed to 24 dyn/cm^2 of fluid shear stress using a cone-and-plate type of shear stress apparatus (Tai et al., 2005).

Detergent-extracted cell model

A monolayer of ECs was washed once with ice-cold TBS(+) (20 mM Tris, pH 7.6, 137 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$), and treated with 0.1% Triton X-100 in TBS(+) supplemented with 1 mM DTT, 0.2 mM ATP, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin on ice with gentle agitation for 90 s After extraction, the detergent-containing buffer was removed and the chamber was gently rinsed once with TBS(+). The model was then incubated with Buffer I (TBS(+) containing 2 mM Na_3VO_4, 0.5 mM ATP, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin) and immediately subjected to stretch or left unstretched at 37°C. For kinase inhibitor studies, immediately after extraction, models were incubated for 1 min at 37°C with inhibitors (stock solutions made in DMSO except for imatinib which was dissolved in water) in Buffer I without ATP and Na_3VO_4. After this pretreatment, 0.5 mM ATP and 2 mM Na_3VO_4 were added and the models were stretched or left unstretched for 5 min at 37°C.

siRNA transfection

Src, Yes, PECAM-1, and scrambled siRNAs were synthesized by Integrated DNA Technologies, whereas Fyn and Fer siRNAs were made by Ambion. Two Fyn siRNA constructs (Fyn-1 and Fyn-2) and their respective sequence-scrambled siRNA constructs were used to establish the biological specificity of Fyn knockdown. ECs were plated 24 h before transfection on collagen-coated silicone chambers at a cell density of ~70–80% confluency. siRNA and Lipofectamine 2000 were diluted in Opti-MEM and added to cells according to the manufacturer's instructions. For controls, cells were treated with Lipofectamine 2000 alone, certified negative control siRNA (QIAGEN), or the scrambled siRNAs described in this section. After 52–56 h of transfection, confluent monolayers were subjected to stretch. Certified negative control siRNA was used at 20 nM. Other siRNA sequences and concentrations used for transfection were the following: human Src, AAGCAACUUGCCCAGCUAUGA (15 nM; Werdich and Penn, 2005); bovine Yes, UUAUGAAGCUAGAACUACA (5 nM; Hirsch et al., 2005); bovine Fyn-1, GGAAGUUUACUCGAUUUCU (10 nM); scrambled siRNA-1, GCUAAGCUACGUUAUUGUU (10 nM); bovine Fyn-2, GGACAAAGAAGCAACAAAA (10 nM); scrambled siRNA-2, GAAAGACGACAAACAAAGA (10 nM); bovine Fer, GGCACUGGGUUUUCAAAUA (20 nM); and bovine PECAM-1, GGAGGAACACAGAGAGAGUUAU (12.5 nM; Tai et al., 2005).

Immunoprecipitation and immunoblotting

Cells were washed with ice-cold PBS, lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM Na_3VO_4, and 1% protease inhibitor cocktail), and the lysate was centrifuged at 18,000 *g* for 15 min at 4°C. 100 µL of the supernatant was incubated with 1 µg of anti–bovine PECAM-1 or anti–human PECAM-1 for 16 h at 4°C with continuous mixing. The immunocomplex was incubated with protein A–conjugated agarose beads (Millipore) for 3 h at 4°C with constant agitation and collected by centrifugation. Beads were washed three times with lysis buffer, and the bound material was eluted with SDS sample buffer, subjected to 8% SDS-PAGE, and electrotransferred to a nitrocellulose membrane. Immunoprecipitated PECAM-1 was immunoblotted with anti-phosphotyrosine (4G10) and anti–PECAM-1 and IRDye-conjugated secondary antibodies. Two-color Western blot fluorescent signals were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Quantification of PECAM-1 phosphorylation and statistics

All the samples to be quantified from a set of experiments were handled together as a unit for immunoprecipitation, SDS-PAGE, and immunoblotting. Using the two-color Western blot technique and the Odyssey software, the intensity of anti–protein phosphotyrosine (4G10) and anti–PECAM-1 immunoblotting was measured on the same blot at the same time. The level of PECAM-1 phosphorylation was determined by the ratio of phosphotyrosine to PECAM-1 staining. The value obtained from unstretched confluent cells was taken as 1 and other values were expressed relative to this. Student's *t* test (one-tailed distribution, equal variance, pairwise comparisons) was used to determine levels of significance. P-values <0.05 were considered to be significant.

Fluorescence microscopy

Cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature. Cell models were fixed similarly but no permeabilization procedure was employed. Antibodies against PECAM-1, protein phosphotyrosine, and pan-cadherin were diluted in PBS containing 0.05% Tween 20. Antibodies against Src, Fyn, and Yes were diluted in TBS containing 2% BSA, and 0.1% Tween 20. Samples were treated with the appropriately diluted primary antibodies for 1 h at room temperature and washed several times with PBS or TBS. Secondary antibodies labeled with Alexa 546 or Alexa 488 were then used to visualize the bound primary antibodies. Control staining was done using either nonimmune IgG as the primary antibody or staining samples with the secondary antibodies alone. Images were observed using an epi-fluorescence microscope (BX51; Olympus) equipped with a camera (RT color Spot; Diagnostic Instruments, Inc.) operated by the camera software (Version 3.5.9.1 for Mac OS), using a 60× 0.9 NA water immersion or 1.4 NA oil immersion lens. Minor image contrast adjustment was done using Photoshop (Adobe).

Scanning electron microscopy

BAECs grown on collagen-coated coverslips were fixed with 2% glutaraldehyde overnight with or without prior detergent extraction. They were then dehydrated, critical-point dried, and coated with gold in a routine

manner at the Electron Microscope Research Core of the University of Rochester School of Medicine and Dentistry and observed in a scanning electron microscope (LEO 982; Carl Zeiss, Inc.).

Online supplemental material

Fig. S1 shows kinetics of total stretch-induced tyrosine phosphorylation in BAECs. Fig. S2 shows PECAM-1 phosphorylation in intact BAECs by cyclic stretch (15%, 1 Hz). Fig. S3 shows PECAM-1 fractionation into the soluble and the cytoskeletal fractions when ECs are extracted. Fig. S4 shows normal expression of Src and Yes in BAECs treated with Fyn siRNA. Fig. S5 shows normal stretch-induced PECAM-1 phosphorylation in BAECs treated with Fer siRNA. Table S1 shows 46 kinases that are inhibited by PP2 by 60% or more. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801062/DC1.

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