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Biochemical and Biophysical Research Communications 320 (2004) 1076–1081

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Role of lateral cell–cell border location and extracellular/transmembrane domains in PECAM/CD31 mechanosensation[☆]

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Received 20 May 2004

11 Abstract

12 Phosphorylation of tyrosine residues on platelet–endothelial cell adhesion molecule-1 (PECAM-1), followed by signal trans-
13 duction events, has been described in endothelial cells following exposure to hyperosmotic and fluid shear stress. However, it is
14 unclear whether PECAM-1 functions as a primary mechanosensor in this process. Utilizing a PECAM-1–null EC-like cell line, we
15 examined the importance of cellular localization and the extracellular and transmembrane domains in PECAM-1 phosphorylation
16 responses to mechanical stress. Tyrosine phosphorylation of PECAM-1 was stimulated in response to mechanical stress in null cells
17 transfected either with full length PECAM-1 or with PECAM-1 mutants that do not localize to the lateral cell–cell adhesion site and
18 that do not support homophilic binding between PECAM-1 molecules. Furthermore, null cells transfected with a construct that
19 contains the intact cytoplasmic domain of PECAM-1 fused to the extracellular and transmembrane domains of the interleukin-2
20 receptor also underwent mechanical stress-induced PECAM-1 tyrosine phosphorylation. These findings suggest that mechano-
21 sensitive PECAM-1 may lie downstream of a primary mechanosensor that activates a tyrosine kinase.

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23 **Keywords:** Platelet endothelial adhesion molecule-1; Endothelial mechanotransduction; Hyperosmotic stress; Fluid shear stress

24 Mechanical stresses, including fluid shear stress (FSS),
25 play an important role in determining endothelial cell
26 (EC) behavior, modulating their physiology, gene ex-
27 pression, and morphology [1,2]. Transfer of FSS forces
28 to the EC first occurs at the luminal cell surface where
29 molecules whose conformations are directly affected by
30 FSS may act as mechanosensors or mechanotransducers.
31 In addition, sites remote from the initial stimulus may act
32 as mechanosensors or mechanotransducers as the force
33 of FSS is transmitted throughout the cell via the cyto-

skeleton [1]. One such location is the lateral cell–cell
adhesion site [3].

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36 Recently, investigators have identified a possible
37 role for platelet–endothelial cell adhesion molecule-1
38 (PECAM-1, CD31) in the sensation of hyperosmotic
39 stress (HOS) and FSS and subsequent signal trans-
40 duction events [4–6]. PECAM-1 is a 130-kDa mem-
41 ber of the immunoglobulin superfamily that is
42 expressed abundantly on the cell surface of ECs,
43 platelets, and many leukocytes. A striking feature of
44 PECAM-1 is its localization at the cell–cell border
45 between adjacent endothelial cells [7,8]. This specific
46 localization may be important to the vascular function
47 of PECAM-1, playing a role in leukocyte transmigra-
48 tion of EC monolayers [9]. In confluent endothelial
49 cells, PECAM-1 molecules on adjacent cells bind
50 homophilically to each other via extracellular domains
51 1 and 2 [10].

[☆] Abbreviation: PECAM-1, platelet–endothelial cell adhesion molecule-1.

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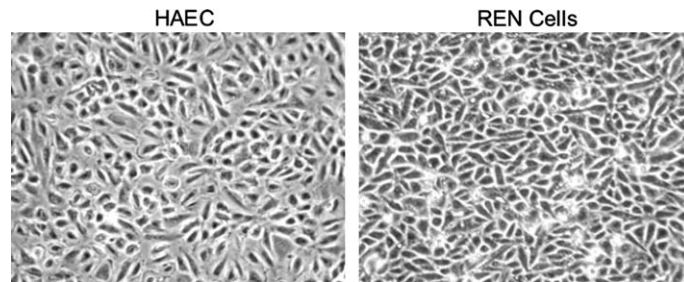


Fig. 1. Morphology of HAEC (left) and REN cells (right) in culture. Cells were cultured on gelatin-covered glass microscope slides and grown to confluence 48 h after seeding. Photomicrographs were obtained just prior to application of shear stress in a parallel plate flow chamber (described in [18]).

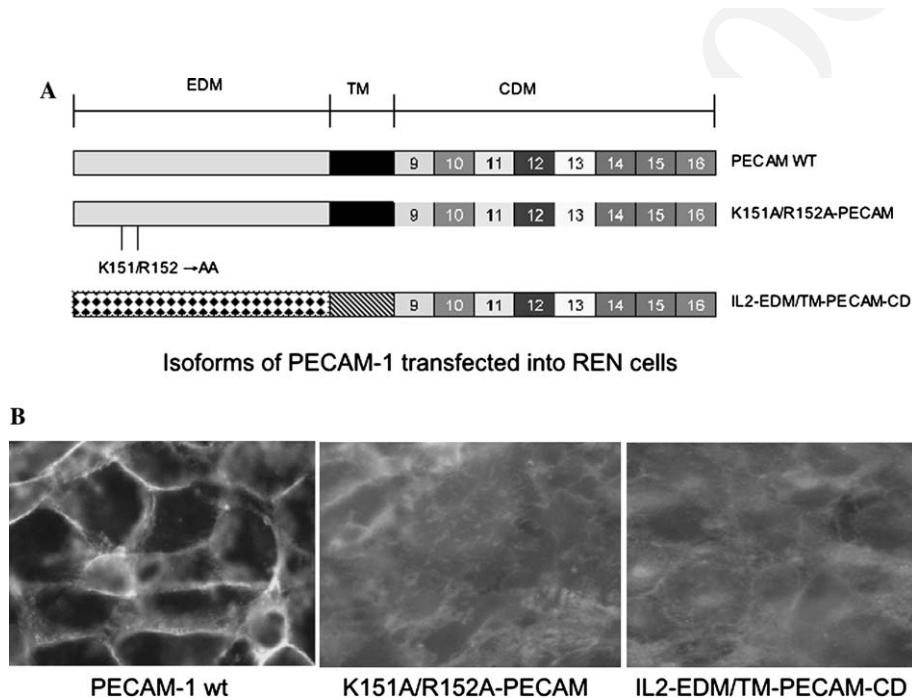


Fig. 2. (A) Isoforms of PECAM-1 transfected into REN cells. (B) Distribution of PECAM-1 isoforms transfected into REN cells. (Left panel) Wild-type PECAM-1 localizes to the lateral cell–cell border. (Center panel) The K151A/R152A-PECAM mutant is found diffusely on the cell membrane, as is the IL2-EDM/TM-PECAM-CD mutant (right panel).

52 REN cells are endothelial-like cells derived from
 53 human malignant mesothelioma [11]. In culture, they
 54 form a confluent monolayer and adopt a “cobblestone”
 55 morphology reminiscent of ECs (Fig. 1). In addition,
 56 REN cells express several surface antigens in common
 57 with ECs but lack PECAM-1; they can be transfected
 58 stably with wild-type or mutant forms of PECAM-1
 59 (Fig. 2A). We have previously used REN cells as an
 60 EC model, finding that many EC signaling processes
 61 may be reconstituted after PECAM-1 expression
 62 [12,13].

63 Utilizing this null cell, we reasoned that if PECAM-1
 64 is a mechanosensor, force-induced PECAM-1 phos-
 65 phosphorylation may require localization to, and organiza-
 66 tion at, the lateral cell–cell border. We also explored
 67 whether the cytoplasmic, extracellular or transmembrane
 68 domains of PECAM-1 are necessary for PECAM-1
 69 mechanosignaling.

Materials and methods

70

Antibodies, reagents, immunoprecipitation, and Western blotting.
 71 Antibodies included the following: mAb 4G6, a murine immuno-
 72 globulin (IgG) directed against the PECAM-1 extracellular Ig loop six
 73 domain [14]; mAb 1.3, a murine IgG directed against the PECAM-1
 74 extracellular domain (a gift of Dr. Peter Newman, Blood Center of
 75 Southeastern Wisconsin, Milwaukee, WI); PCD, a rabbit polyclonal
 76 antibody directed against the PECAM-1 cytoplasmic domain; ab8325
 77 (Abcam, Cambridge, UK), a murine mAb directed against the
 78 α -subunit of the interleukin-2 receptor (IL2R); anti-SHP-2 mAb (Cell
 79 Signaling Technology, Santa Cruz, CA); and PY20 (Transduction
 80 Laboratories, BD Biosciences, Palo Alto, CA), an anti-phosphotyrosine
 81 rabbit polyclonal Ab. Purified antibodies were obtained by protein
 82 G affinity chromatography of hybridoma supernatants or serum.
 83 Active binding of antibodies was confirmed by flow cytometry.
 84

For immunoprecipitation, thawed lysates were preabsorbed with
 85 protein A-conjugated Sepharose beads (Amersham–Pharmacia). After
 86 removal from the beads, the precleared supernatants were transferred
 87 to fresh microfuge tubes and immunoprecipitated by incubation with
 88 mAb 4G6 (for WT PECAM-1 and the K151/R152A mutant) or
 89

90 ab8325 (for the IL2PCD construct), followed by incubation with
91 protein A-conjugated Sepharose beads.

92 Lysates were then separated on 4–12% gradient SDS–polyacryl-
93 amide reducing gels (Invitrogen) and transferred to PVDF membranes
94 (Millipore). Membranes were probed with mAb 1.3 or pAb PCD and
95 then counterstained with HRP-conjugated donkey anti-mouse IgG
96 (Cappel) or HRP-conjugated goat-anti-rabbit IgG (Jackson), and
97 signals were visualized with ECL (Amersham–Pharmacia). Mem-
98 branes were then stripped in a buffer containing 62.5 mM Tris–HCl
99 (pH 6.8), 2% SDS, and 100 mM of 2-mercaptoethanol, then reprobed
100 with PY20 and counterstained with HRP-conjugated goat-anti-rabbit
101 IgG. Signals were again detected by ECL. Images were captured on a
102 desktop scanner (Canon CanoScan D1250U2F) utilizing Adobe
103 Photoshop 7.0.

104 *Cell lines and mutant PECAM-1 constructs.* Human aortic endo-
105 thelial cells (HAEC, Clonetics) were cultured in endothelial basic
106 medium-2 (EBM-2, Clonetics) containing 2% fetal bovine serum and
107 Bullet kit reagents (Clonetics). Only HAECs between passages 2 and 6
108 were used.

109 REN cells, a human mesothelioma cell line previously isolated in
110 our laboratories [11], were grown in RPMI (Gibco) supplemented with
111 10% FBS and 2 mM L-glutamine (R10 media) containing 10,000 U
112 penicillin and 10,000 U streptomycin. PECAM-1 mutant constructs
113 IL2PCD and K151/R152A [15] as well as wild-type PECAM-1 were
114 subcloned into the pcDNA-neo vector and transfected into REN cells.
115 Expression was subsequently confirmed by flow cytometry (Coulter) as
116 described previously [12]. Stable polyclonal populations of REN cell
117 transfectants were generated by bead sorting (Dynal) and selected in
118 G418 (0.5 mg/mL) supplemented R10 media as previously described
119 [13].

120 The IL2PCD PECAM-1 mutant contains the extracellular and
121 cytoplasmic domains of the interleukin-2 receptor fused to the full
122 cytoplasmic domain of PECAM-1 [15]. The K151/R152A mutant
123 contains mutations lysine–arginine (KR) at amino acid positions 151
124 and 152 to alanine–alanine in the putative glycosaminoglycan binding
125 region of PECAM-1 (amino acids 149–155, see [16]). Previously, these
126 mutant forms of PECAM-1 have been demonstrated to spread dif-
127 fusely over the cell surface rather than localize to lateral cell–cell
128 adhesion junctions, and we confirmed these observations in the cell lines
129 used for these experiments (Fig. 2B) [15].

130 *Immunofluorescent staining.* Cells were grown on gelatin-coated
131 coverslips, washed in phosphate-buffered saline (PBS), fixed with 3%
132 paraformaldehyde for 20 min, and then permeabilized with ice-cold
133 0.5% NP-40 for 1 min. After washing, cells were stained using anti-
134 PECAM-1 mAb 4G6 and polyclonal antibody “PCD” (directed
135 against the cytoplasmic domain of PECAM-1) as previously described
136 [17]. Cells were viewed on a Nikon eclipse E400 fluorescence micro-
137 scope using a 40× oil fluorescence lens and photographed with a
138 Nikon Coolpix 4500 digital camera.

139 *Hyperosmotic stress and fluid shear stress.* Cells were seeded onto
140 gelatin-coated glass microscope slides 48 h prior to the experiment
141 and grown to confluence. For experiments with HAECs, EBM-2
142 (Clonetics) containing 2% fetal bovine serum and Bullet kit reagents
143 (Clonetics), supplemented with 1% dextran, was used. To enhance the
144 PY-PECAM-1 signal, this medium was supplemented with 5 mM
145 NaVO₃ (pH 7.4); HAECs incubated for 3 h in NaVO₃-containing
146 medium were used as a positive PY-PECAM-1 control.

147 REN cells were incubated in R10 medium containing 1% dextran
148 and 5 mM NaVO₃ (pH 7.4) at 37°C for 2 h prior to exposure to me-
149 chanical stress. For FSS, glass slides were placed in a parallel flow
150 chamber [18] and subjected to 13 dyn/cm² of continuous shear stress
151 for 15 min with cell growth media supplemented with 1% dextran (to
152 increase the media’s viscosity) and 5 mM NaVO₃. For HOS, cells were
153 exposed to medium containing 1% dextran, 5 mM NaVO₃, and
154 600 mM sucrose. After mechanical stress, cells were washed twice with
155 ice-cold PBS containing 1 mM NaVO₃ and lysed for 20 min on ice with
156 a buffer containing 0.01 M Tris–acetate (pH 8.0), 0.5% NP-40, 0.5 mM

Ca²⁺, 10 mcg/mL leupeptin, 10 mcg/mL aprotinin, 2 mM PMSF, and
2 mM NaVO₃. Lysates were centrifuged at 14,000g for 10 min at 4°C
and the supernatant was stored at –80°C.

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Results and discussion

160

Fluid shear stress leads to tyrosine phosphorylation of PECAM-1 in HAECs and REN cells transfected with wild-type PECAM-1

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FSS and HOS induce tyrosine phosphorylation of
PECAM-1 in cultured bovine aortic endothelial cells
[5,6]. To confirm this observation, and to ascertain
whether this phenomenon is present in human EC,
physiologic FSS (13 dyn/cm²) was applied for 15 min, or
HOS for 10 min, to cultured HAECs. PECAM-1 puri-
fied from HAECs subjected to FSS demonstrated sig-
nificantly higher levels of tyr-P than controls (Fig. 3).
We also confirmed the observation that PECAM-1–tyr-
P co-immunoprecipitated with SHP-2, as observed by
other investigators (data not shown) [19].

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In order to explore the role of PECAM-1 as a
mechanosensitive molecule in depth, we chose the REN
cell model as a null cell. Because wild-type-PECAM-1 is
expressed abundantly on all known lines of ECs, de-
tecting the effects of mutations to PECAM-1 is difficult.
Some investigators have employed anti-sense s-oligo
techniques to knock down the expression of wt-PE-
CAM-1, but such techniques only suppress the expres-
sion of wt-PECAM-1 to approximately 70% of normal
[5]. Thus, we subjected REN cells transfected with wt-
PECAM-1 (REN-HP) to FSS and HOS. In order to
strengthen the PECAM-1–tyr-P signal in Western blot-
ting, phosphatase activity was inhibited by incubating
the cells with growth media containing 5 mM NaVO₃ for
2 h prior to FSS or HOS (control samples were incu-
bated with growth media containing 5 mM NaVO₃ for
2 h 15 min). FSS or HOS stimulated tyr-P of PECAM-1

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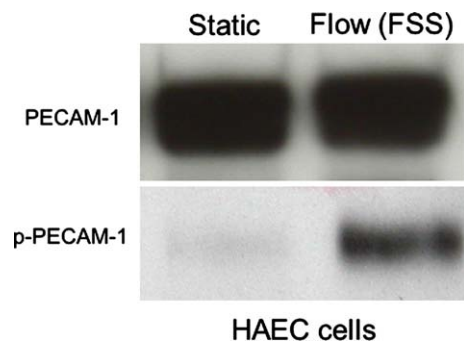


Fig. 3. Three hour incubation with 5 mM sodium vanadate and 15 min of fluid shear stress (FSS) lead to tyrosine phosphorylation of PECAM-1 in HAECs. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 mAb 1.3 (upper panel). Membranes were then stripped and reprobed with anti-PY pAb PY20 (lower panel).

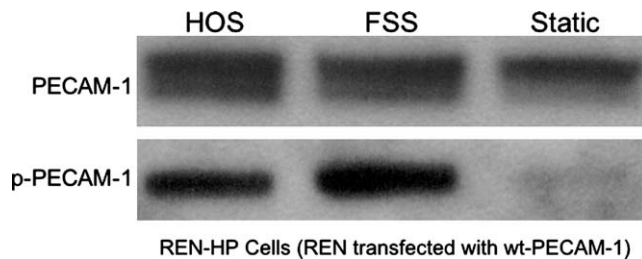


Fig. 4. HOS and 13 dyn/cm² FSS lead to tyrosine phosphorylation of PECAM-1 in REN-HP cells. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 mAb 1.3 (upper panel), stripped and reprobed with anti-PY pAb PY20 (lower panel).

192 in REN-HP cells when compared with static control
193 (Fig. 4). In Western blotting of lysates of REN cells, a
194 negative control lacking PECAM-1, as expected, no
195 corresponding band was visible (data not shown).

196 *Homophilic PECAM-1 binding is not required for*
197 *mechanically induced tyrosine phosphorylation*

198 In a study of mechanically induced PECAM-1-tyr-P,
199 Osawa et al. [6] proposed a model in which mechanical
200 force acts directly on PECAM-1, causing a conformational
201 change that permits tyr-P of the cytoplasmic domain of
202 PECAM-1. To explore whether lateral cell-cell
203 adhesion site localization and homophilic binding between
204 PECAM-1 molecules on adjacent cells are required for
205 force-induced PECAM-1-tyr-P, REN cells stably
206 expressing the K151/R152A mutant form of PECAM-1
207 were exposed to 15 min of FSS or 10 min of
208 HOS. In previous work, we noted that this mutant form
209 of PECAM-1 does not localize to the lateral cell-cell
210 border (Fig. 2) and does not support homophilic binding
211 [15]. FSS and HOS stimulated increased PECAM-1-
212 tyr-P in REN cells transfected with the K151/R152A
213 mutant form of PECAM-1 (Fig. 5), demonstrating that
214 membrane localization and homophilic binding between
215 confluent cells are not required for mechanosignaling
216 responses.

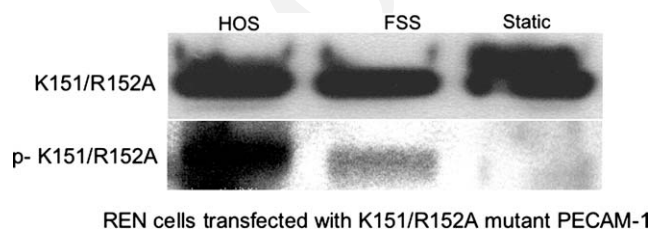
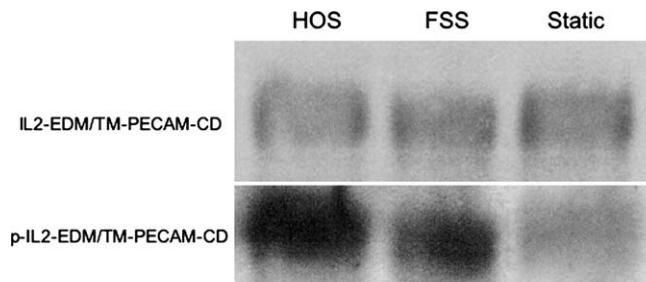


Fig. 5. HOS and 13 dyn/cm² FSS induce tyrosine phosphorylation of PECAM-1 in REN cells transfected with the K151/R152A mutant of PECAM-1. Cell lysates were immunoprecipitated with anti-PECAM-1 mAb 4G6 and membranes were blotted with anti-PECAM-1 pAb PCD (upper panel), stripped, and reprobed with anti-PY pAb PY20 (lower panel).

The extracellular and transmembrane domains of 217
PECAM-1 are not required for mechanically induced 218
tyrosine phosphorylation 219

To explore the importance of the extracellular and 220
transmembrane domains of PECAM-1 in mechanosen- 221
sation, we exposed REN cells transfected with mutant 222
forms of PECAM-1 to FSS. In previous experiments, we 223
have demonstrated that when a mutant form of PE- 224
CAM-1 containing the non-homologous IL2R extra- 225
cellular and transmembrane domains fused to the intact 226
PECAM-1 cytoplasmic domain (IL2PCD mutant) is 227
transfected into REN cells, it is expressed diffusely 228
throughout the cell membrane (Fig. 2), but continues to 229
serve as a substrate for c-Src-dependent, H₂O₂-induced 230
PECAM-1-tyr-P [15,20]. In addition, this PECAM-1 231
mutant regulates H₂O₂-induced cation channel activity 232
with kinetics identical to that of wt-PECAM-1 [20]. 233
REN cells stably transfected with the IL2PCD mutant 234
of PECAM-1 were exposed to FSS and HOS as de- 235
scribed above in the presence of phosphatase inhibition 236
by vanadate. Both forms of mechanical stress resulted in 237
increased PECAM-1-tyr-P in REN cells transfected with 238
the IL2PCD mutant form of PECAM-1 (Fig. 6). 239

After confirming that native PECAM-1 undergoes 240
tyrosine phosphorylation in response to mechanical 241
stress in human endothelial cells, we have reproduced 242
the phenomenon in endothelium-like REN cells trans- 243
fected with PECAM-1. Since this is a null cell, it per- 244
mitted an investigation of altered protein structure and 245
cellular localization in PECAM mechanosensing by 246
transfection of PECAM-1 mutant constructs. Lateral 247
cell-cell border localization is not required for force- 248
induced PECAM-1 tyrosine phosphorylation. Osawa 249
et al. [6] demonstrated a similar finding in sparsely cul- 250
tured cells. Our work extends this finding to the highly 251
structured confluent monolayer, a situation found in 252
vivo and reproduced in both endothelial and REN in 253
vitro, where homophilic binding occurs between PE- 254
CAM-1 molecules on adjacent cells. Not only does 255
force-induced PECAM-1-tyr-P appear to be independ- 256
ent of PECAM localization to the lateral membrane, 257
but it appears that neither the extracellular nor trans- 258
membrane domains are necessary for mechanosignaling. 259
The mutant forms of PECAM-1-tyr-P also associated 260
with the phosphatase SHP-2, as shown by other inves- 261
tigators [19] indicating all transfected forms of PECAM- 262
1 to be a substrate for a tyrosine kinase in the present 263
study. The evidence suggests that mechanosensors may 264
activate a tyrosine kinase that in turn phosphorylates 265
the cytoplasmic domain of PECAM-1, leading to SHP2 266
activation and eventually Erk-1/2 activation. It has been 267
suggested that PECAM-1 may regulate or associate with 268
other potentially mechanoresponsive molecules: β -cate- 269
nin [21], focal adhesion kinase [22], and integrin $\alpha_v\beta_3$ 270
[23,24]. Whether the mechanically induced behavior of 271



REN Cells transfected with mutation of PECAM-1 containing only the cytoplasmic domain of wild type and the transmembrane and extracellular domains of interleukin-2.

Fig. 6. HOS and 13 dyn/cm² FSS lead to tyrosine phosphorylation of PECAM-1 in REN cells transfected with the IL2PCD mutant of PECAM-1. Cell lysates were immunoprecipitated with anti-IL2R mAb and membranes were blotted with anti-PY pAb PY20 (lower panel). Membranes were then stripped and reprobbed with anti-PECAM-1 pAb PCD (upper panel).

272 these molecules modulates or is modulated by PECAM-
273 1 has not been elucidated.

274 Mechanical stress is an important determinant of
275 endothelial cell behavior [1,2]. Areas of disturbed flow,
276 for example, are more prone to atheroma formation
277 [25,26]. Abnormal mechanical stress may also play a
278 role in the pathogenesis of pulmonary hypertension
279 [27], ventilator-induced lung injury [28,29] or glomer-
280 ulonephropathy [30]. PECAM-1 is expressed abun-
281 dantly on endothelial cells, platelets, and most
282 leukocytes. It is believed to play a role in mediating
283 adhesion between adjacent endothelial cells, angiogen-
284 esis, and neutrophil adhesion to, and migration
285 through, the endothelial monolayer [9,31,32]. PECAM-
286 1-null mice, however, do not display developmental
287 abnormalities or significant vascular defects, although
288 bleeding time is increased, leukocyte transendothelial
289 migration is slowed, and the blood–brain barrier may
290 be weakened [33–35].

291 In summary, utilizing EC-like REN cells stably
292 transfected with wild-type and mutant PECAM-1
293 constructs to elucidate which domains of PECAM-1
294 confer mechanosensitivity, we demonstrate that in the
295 confluent monolayer, phosphorylation does not depend
296 upon lateral membrane localization of the protein and
297 cell–cell homophilic PECAM-1 binding. The trans-
298 membrane and extracellular domains of PECAM-1 are
299 not necessary for mechano-responsiveness. The kinase,
300 which remains to be identified, appears to be activated
301 by a more direct effect of mechanical stress on the
302 cells.

303 Acknowledgments

304 We gratefully acknowledge the technical assistance of Rebecca
305 Riley and thank Dr. Peter Newman of the Medical College of Wis-
306 consin for mAb1.3. This work was supported by T32 training Grant
307 HL07954 (D.A.K.) and by NIH Grants HL64388, HL62250, and
308 HL71174.

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