Supporting Information

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SI Text

Reagents. α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Human APC and active site–blocked APC-DEGR (dansyl-EGR chloromethyl ketone) were obtained from Hematologic Technologies (Essex Junction, VT). The peptides agonists, TFLLRNPNDK and SLIGKV, were synthesized at the UNC Peptide Facility (Chapel Hill, NC). Hirudin and actin antibody were obtained from Sigma (St. Louis, MO). Caveolin-1 antibody was from BD Biosciences (San Jose, CA). Horseradish peroxidase (HRP)–conjugated goat anti-mouse and -rabbit secondary antibodies were from Bio-Rad (Richmond, CA).

EA.hy926 Cells Expressing PAR1 shRNA or CAV1 shRNA. The short hairpin RNAi (shRNA) 5′-AGAUUAGUCUCCAUCAAUA-3′ targeting PAR1 and nonspecific siRNA 5′-CUACGUCCAGGAGCGCACC-3′ were subcloned into *pSilencer* 5.1-U6 Retro (Ambion, Austin, TX) as described elsewhere (1). The caveolin-1 shRNA (5′-AAGATGTGATTGCAGAACCAGA-3′) construct in pRVH1-puro was obtained from K. Simons (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) (2) and scrambled shRNA 5′-GTAAATGCCATACCTTATA-3′ of PAR1 siRNA sequence was inserted into pSUPER.retro.puro vector (Oligoengine, Seattle, WA). Retroviruses were generated using PA317 packaging cells and used to infect EA.hy926 cells. Mass populations of cells stably transduced with PAR1 shRNA, caveolin-1 shRNA, nonspecific scrambled shRNAs, and vector control constructs were selected with 0.6 μg/ml of puromycin.

PAR1 Phosphorylation. To assess PAR1 phosphorylation, EA.hy926 endothelial cells were labeled with 200 μCi [^{32}P]orthophosphate (Perkin-Elmer, Boston, MA) in phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml bovine serum albumin (BSA) for 3 hours at 37 °C. After cell treatments, PAR1 was immunoprecipitated with anti-PAR1 WEDE-15 monoclonal antibody, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to membranes. Phosphorylated receptor was detected by autoradiography. The amount of PAR1 in immunoprecipitates was determined by immunoblotting with polyclonal anti-PAR1 antibody.

Permeability Assay. Endothelial barrier permeability was quantified by measuring the flux of Evans blue-bound BSA (Sigma, St. Louis, MO) as previously described (3). Briefly, EA.hy926 cells (5×10^4 cells per well) were plated on 12-mm-diameter transwell dishes coated with 2% gelatin (3-μm pore size polycarbonate filter (Costar, Corning, Ithaca, NY) and grown for 4–6 days at 37 °C. The upper and lower chambers contained 500-μl and 1500-μl growth medium, respectively. The day before the experiment, the growth medium was replaced with starvation medium (DMEM containing 0.2% fetal bovine serum [FBS]). On the day of the experiment, cells were washed and then incubated with or without 10 nM APC for 3 hours at 37 °C, added to the upper chamber. Cells were washed and then incubated with or without 10 nM thrombin or 10 nM APC for 10–20 minutes at 37 °C added to the upper chamber. The medium in the upper chamber was then replaced with 0.67 mg/ml Evans blue–BSA diluted in growth medium containing 4% BSA (Sigma) and after 10–20 minutes the optical density (OD) at 650 nm was measured in a 1:3 diluted 50-μl sample from the lower chamber using a Molecular Devices Plate Reader (Sunnyvale, CA).

RhoA and Rac1 Activity Assays. GST-rhotekin RBD and PAK-PBD were purified as described (4–6) and assays were conducted as described below. EA.hy926 endothelial cells were plated in six-well dishes at 5×10^5 cells per well, grown for 2 days, deprived of serum, and then treated with or without agonists for various times at 37 °C. To assess RhoA activation, cells were lysed in buffer containing 50 mM Tris-HCL, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, and 20 mM $MgCl_2$ with protease inhibitors. Endogenous RhoA activity was then measured in pull-down assays using a GST fusion of the Rho binding domain (RBD) of Rhotekin. To monitor Rac1 activity, cells were lysed in buffer containing 50 mM Tris-HCL, pH 7.6, 150 mM NaCl, 1% Triton X-100, 20 mM $MgCl_2$ with protease inhibitors, and then incubated with GST-p21-activated kinase (PAK-1) binding domain (PBD) fusion protein. The GST PAK-PBD and Rhotekin-RBD fusion constructs were transformed into BL21 (DE3) *Escherichia coli,* and fusion proteins were induced and prepared using standard techniques. GST-PAK-PBD (120 μg) or Rhotekin-RBD (90 μg) bound to glutathione-Sepharose beads were then incubated with cell lysates for 1 hour at 4 °C and washed. GTP-bound RhoA or Rac1 were eluted in 2X SDS-sample buffer containing 100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, resolved by SDS-PAGE, and transferred to membranes. The amount of endogenous activated RhoA and Rac1 were then detected by immunoblotting using a monoclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal anti-Rac1 antibody from BD Biosciences, respectively. Immunoblots were developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Arlington, IL), imaged by autoradiography, and quantitated using a Bio-Rad Fluor-S- MultiImager (Richmond, CA).

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2. Schuck S, Manninen A, Honsho M, Fullekrug J, Simons K (2004) Generation of single and double knockdowns in polarized epithelial cells by retrovirus-mediated RNA interference. *Proc Natl Acad Sci USA* 101:4912–4917.
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4. Yohe MF, Rossman KL, Gardner OS, Karnoub AE, Snyder JT (2007) Auto-inhibition of the Dbl family protein Tim by an N-terminal helical motif. *J Biol Chem* 282:13813–13823.
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6. Arthur WT, Burridge K (2001) RhoA inactivation by p190RhoGAP regulates cell spreading and migration promoting membrane protrusion and polarity. *Mol Biol Cell* 12:2711–2720.

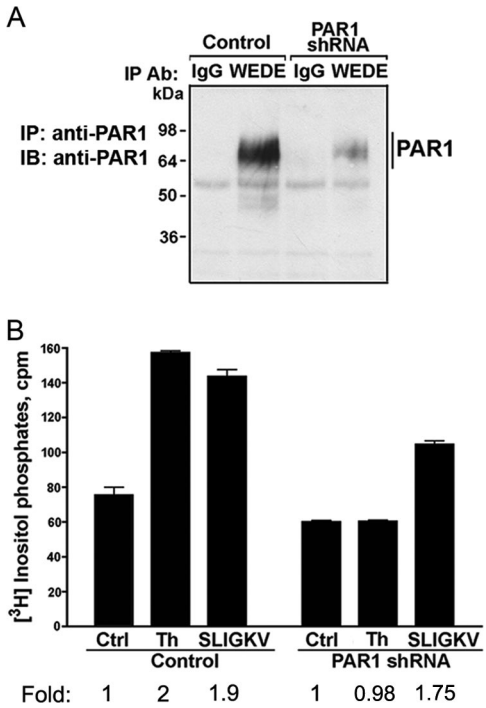

Fig. S1. Thrombin signaling is lost in EA.hy926 endothelial cells expressing PAR1 shRNA. (*A*) Equivalent amounts of lysates prepared from control and PAR1 shRNA–expressing cells were immunoprecipitated with monoclonal anti-PAR1 antibody or IgG control. Immunoprecipitates were immunoblotted with anti-PAR1 polyclonal antibody to detect PAR1 expression. (*B*) Control and PAR1-deficient endothelial cells labeled with *myo*-[^{3}H]inositol were incubated in the presence or absence of 10 nM thrombin (Th) or 100 μM SLIGKV (PAR2 agonist peptide) for 60 minutes at 37 °C in medium containing lithium chloride. The amounts of accumulated [^{3}H]IPs were then measured. Data are shown as total [^{3}H]inositol phosphates (cpm) accumulated and expressed as fold-increase over untreated control.

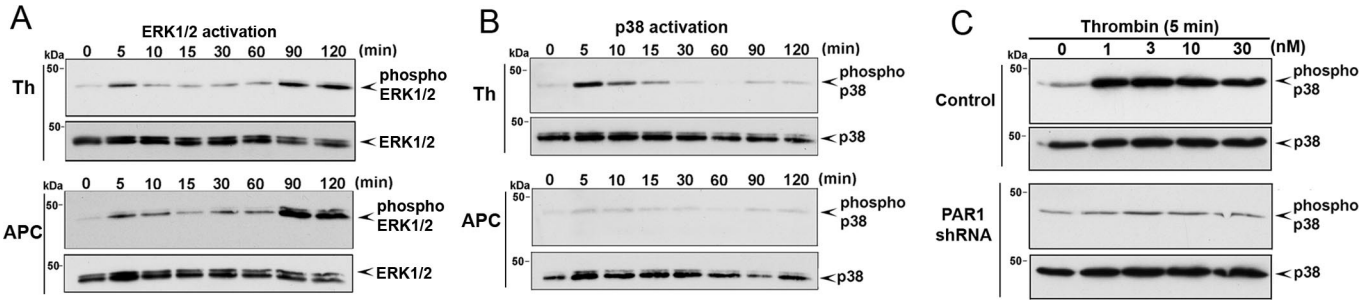

Fig. S2. Activation of ERK1/2 and p38 signaling by thrombin and APC. (*A* and *B*) Serum-starved endothelial cells were incubated in the absence or presence of 10 nM thrombin (Th) or 10 nM APC (with 0.5 U/ml hirudin) for various times at 37 °C. ERK1/2 and p38 activation were then determined using specific anti-phospho MAPK antibodies. Membranes were stripped and reprobed for total MAPK. (*C*) Serum-starved control and PAR1-deficient cells were incubated with or without various concentrations of thrombin (Th) for 5 minutes at 37 °C. Cells were lysed, and activation of p38 was determined using anti-phospho p38 antibody. Membranes were reprobed with total p38 antibody to control for loading.

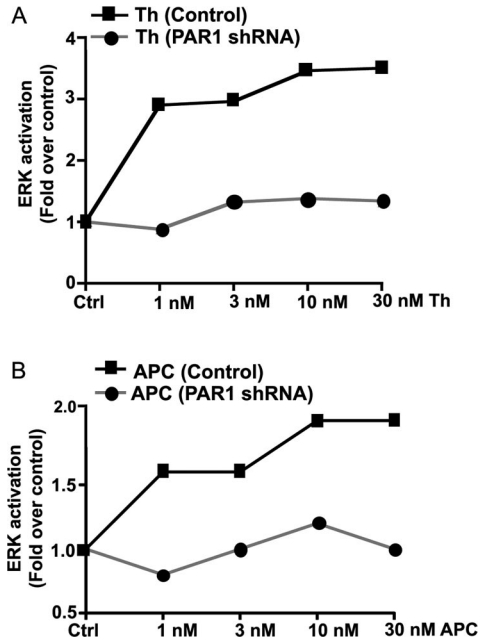

Fig. S3. PAR1 is essential for thrombin and APC signaling in EA.hy926 endothelial cells. Immunoblots shown in Figs. 1*A* and 1*B* were quantified by densitometry using a Bio-Rad Fluor S-MultiImager (Richmond, CA). Data shown are expressed as fold-increase over control of the representative experiment shown in Fig. 1.

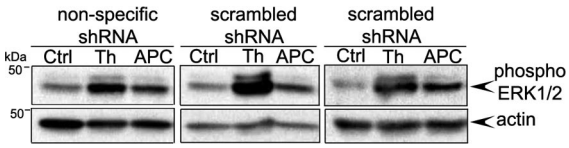

Fig. S4. Thrombin and APC signaling in nonspecific and scrambled shRNA expressing EA.hy926 endothelial cells. Serum-starved EA.hy926 cells stably transduced with nonspecific or scrambled shRNA were incubated in the absence or presence of 10 nM thrombin (Th) or 10 nM APC (0.5 U/ml hirudin) for 5 minutes at 37 °C. Cells were lysed and ERK1/2 activity was determined by immunoblotting. Membranes were stripped and reprobed with an anti-actin antibody to control for loading. Data shown are representative of several independent experiments.

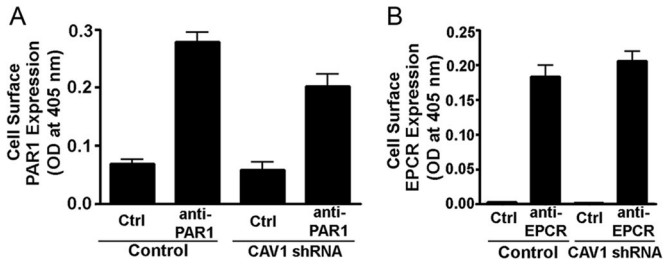

Fig. S5. PAR1 and EPCR cell surface expression. (*A* and *B*) Control and caveolin-1 (CAV1)–deficient endothelial cells were fixed, and the amounts of cell surface PAR1 and EPCR at steady state were determined by ELISA. Data (mean ± SD, $n = 3$) are representative of replicate experiments.

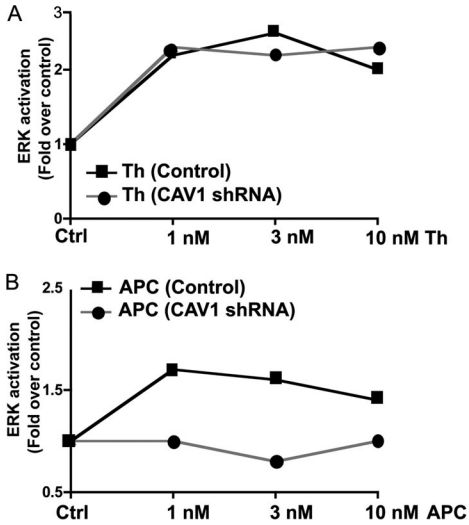

Fig. S6. Caveolin-1 is critical for APC but not thrombin activation of PAR1 signaling. Immunoblots shown in Figs. 3*B* and 3*C* were quantified by densitometry using a Bio-Rad Fluor S-MultiImager (Richmond, CA). Data shown are expressed as fold-increase over control of the representative experiment shown in Fig. 3.

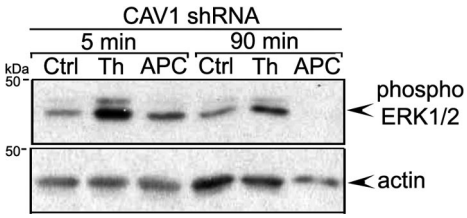

Fig. S7. APC signaling is lost in caveolin-1–deficient endothelial cells. Serum-deprived caveolin-1 shRNA expressing EA.hy926 endothelial cells were incubated with 10 nM thrombin or 10 nM APC (0.5 U/ml hirudin) for 5 minutes or 90 minutes at 37 °C. Cells were lysed and ERK1/2 activity was determined by immunoblotting. The membranes were stripped and reprobed with an anti-actin antibody to control for loading. Similar results were observed in multiple independent experiments.

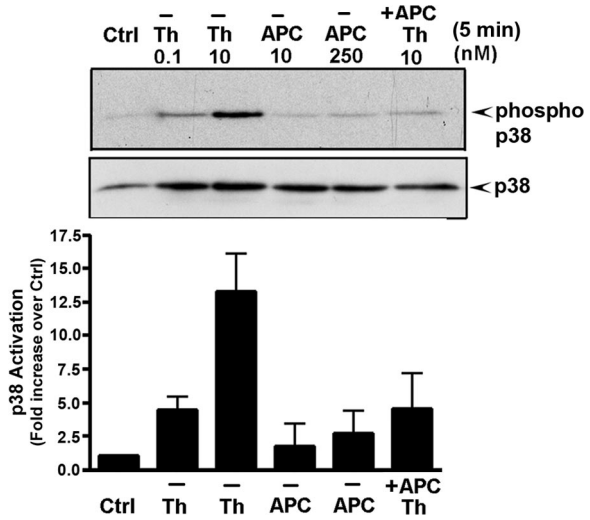

Fig. S8. Desensitization of thrombin-stimulated p38 activation after APC pretreatment. Serum-deprived endothelial cells were pretreated with 10 nM APC (with 0.5 U/ml hirudin) for 1 hour at 37 °C, washed, and then incubated in the absence or presence of various concentrations of thrombin (Th) or APC (0.5 U/ml hirudin) for 5 minutes at 37 °C. Cells were lysed, and the activation of p38 was determined using anti–phospho-p38 antibodies. Membranes were stripped and re-probed with anti-p38 antibody to control for loading. Data (mean ± SE) shown are expressed as the fold-increase over control and are the averages of three independent experiments.

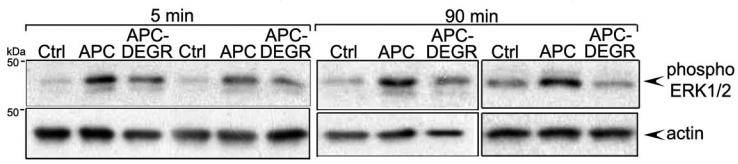

Fig. S9. Signaling by APC and active site–blocked APC-DEGR in endothelial cells. Serum-starved wild-type EA.hy926 endothelial cells were incubated in the absence or presence of 10 nM APC or active site–blocked 10 nM APC-dansyl-EGR chloromethyl ketone (DEGR) for 5 minutes or 90 minutes at 37 °C. Cells were lysed and ERK1/2 activity was determined by immunoblotting. Membranes were stripped and re-probed with anti-actin antibody as a control for loading. These findings were observed in several separate experiments.