Materials and Methods

Platelet isolation and aggregation

Pooled platelet concentrates in plasma (platelet concentrates from 5 donors in ABO blood-group-matched plasma) were obtained from the Dutch Bloodbank (Sanquin). First, platelet concentrates were centrifuged for 20 minutes at 120g, to remove remaining red and white blood cells. Next, platelets were washed twice with isolation buffer (36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 5.6 mM D-glucose, pH 6.5, containing 0.35% [w/v] bovine serum albumin (BSA)) by centrifugation for 10 minutes at 2,000g. Platelets were washed once with Tyrode's Solution (Sigma), centrifuged for 10 minutes at 2,000g and resuspended to a final concentration of 1×10^9 platelets/mL in Tyrode's Solution. Platelet aggregation was measured using a ChronoLog 700 Aggregation System. Platelets were pre-incubated for 10 minutes with vorapaxar (Axon Medchem BV), parmodulin-2 (Axon Medchem BV) or vehicle (DMSO). Platelet aggregation was triggered with 10 µM thrombin-TL peptide SFLLRN-NH2 (Peptides international). Normalized response was calculated per sample by normalizing the maximal aggregation to the maximal aggregation in the vehicle control. Data were fitted using "log(inhibitor) vs. normalized response -- Variable slope" and IC_{50} values were calculated (Graphpad Prism version 6.04). To determine specificity of vorapaxar and parmodulin-2 for PAR1, platelets were pre-incubated for 10 minutes with 100 nM vorapaxar, 100 µM parmodulin-2, or vehicle (DMSO). Platelet aggregation was triggered by thrombin-TL peptide (10 µM), PAR4 peptide AYPGKF-NH2 (150 µM) (Peptides international), Collagen (2 µg/ml) (Chronolog) or Thromboxane A_2 receptor agonist U466619 (5 µM) (Tocris). Statistical analysis was performed using two-way ANOVA with post hoc Tukey's multiple comparison test (Graphpad Prism version 6.04).

Transendothelial electrical resistance

Blood Outgrowth Endothelial Cells (BOECs) were isolated as described previously.[1] Cells were pooled from 3 donors in a 1:1:1 ratio and cultured in EGM-2 (Lonza) containing 20% Fetal Bovine Serum (FBS). BOECs from passage 6 – passage 10 were used for the transendothelial electrical resistance (TER) experiments. Although TER represents only one aspect of the endothelial barrier function[2], this technique is widely used to study the thrombin response in ECs. Golden electrodes (8W10E, Applied BioPhysics Inc.) were treated with 10 mM L-Cysteine and subsequently coated with 50 µg/ml collagen type I (BD Biosciences). Next, BOECs were seeded (80,000 cells/array) and grown to confluence o/n, TER was measured every 3 minutes at 30 kHz at 37°C, 5% CO_2 using an Electric Cell-substrate Impedance Sensing system model 9600 (Applied BioPhysics Inc.). Cells were pre-incubated for 1 hour with vorapaxar, parmodulin-2 or vehicle (DMSO), stimulated with 10 nM, 50 nM or 100 nM thrombin (1U/ml thrombin = 10 nM) (1500-3000 National Institute of Health(NIH) units/mg protein, T4393, Sigma) and TER was monitored for 150 minutes. For each time point, values were normalized to the resistance 9 minutes prior to thrombin addition. Normalized response was calculated per sample by normalizing the minimal resistance after thrombin addition to the minimal resistance after thrombin addition in the vehicle control. Data were fitted using "log(inhibitor) vs. normalized response -- Variable slope" and IC_{50} values were calculated (Graphpad Prism version 6.04).

Endothelial cell culture and SILAC labeling

For Stable Isotope Labeling with Amino acids in Cell culture (SILAC) labeling, pooled BOECs from 3 donors were cultured as described with minor modifications.[3] Briefly, BOECs were pooled form 3 donors in a 1:1:1 ratio and cultured in SILAC EBM-2 (w/o Arginine and Lysine, custom made, Lonza) supplemented with bullet kit, 20% FBS (passage 4-6) or 1kD in house dialyzed FBS (passage 7-8), and Lysine 0 and Argine0 (Light) (Sigma), or Lysine4 and Argine6 (Medium), or Lysine8 and Argine10 (Heavy) (Cambridge Isotopes). SILAC incorporation was fully established after 5 passages (start labeling: passage 4, final stage: passage 8). For the thrombin/PAR1 inhibitor dataset: Prior to the experiment, cells were serum starved for 2h in SILAC EBM-2 and pre-incubated for 1 hour with 100nM vorapaxar, 10 µM parmodulin-2 or vehicle (DMSO). Next, cells were stimulated with 50 pM (0.005U/ml and 50 pM) or 10 nM thrombin (1U/ml thrombin = 10 nM), or 10µM thrombin-TL peptide for 2 or 10 minutes (1x15 cm culture dish per condition). For the tethered ligand peptides dataset: Prior to the experiment, cells were serum starved for 2h in SILAC EBM-2. Next, cells were stimulated with 50 µM tethered ligand peptide corresponding to cleavage of the PAR1 N-terminus by the following proteases: Neutrophil Protease 3 (PR3-TL: TLDPRSF-NH2), Matrix metalloprotease-1 (MMP1-TL: PRSFLLRN-NH2), Thrombin (Thrombin-TL: SFLLRN-NH2), Neutrophil elastase (NE-TL: RNPNDKYEPF-NH2) or Activated protein C (APC-TL: RNPNDKYEPF-NH2) (custom peptide synthesis, Biosynthesis) for 2 or 20 minutes (1x15 cm culture dish per condition). For the APC/SEW2871 stimulation: Prior to the experiment, cells were serum starved for 2h in SILAC EBM-2. Next, cells were stimulated with 20 nM APC (Haematologic Technologies) + 1 U/ml hirudin (Sigma), 5 µM SEW2871 (Cayman chemical) or 1 U/ml hirudin alone for 2 or 10 minutes (1x15 cm culture dish per condition). Cells were washed once with Hanks Balanced Salt Solution (HBSS, Invitrogen) and lysed in SDS lysis buffer (4% SDS, 100 mM ditiotreitol (DTT), 100 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, supplemented with phosphatase and protease inhibitor cocktail (Thermo Scientific)). Next, cell lysates were incubated for 5 minutes at 95 °C, sonicated and centrifuged for 10 minutes at 16,000g. The cleared lysates were snap-frozen and stored at -80 °C. Experiments were performed in triplicate, where the three different labeling conditions were swapped.

Proteome analysis and phosphopeptide isolation

Proteome analysis and phosphopeptide isolation were performed as described previously with minor modifications.[3] Briefly, light, medium and heavy SILAC-labeled cell-lysates were mixed together in a 1:1:1 ratio. Mixed proteins were reduced, alkylated and digested with trypsin using the FASP method.[4] For proteome analysis, digested peptides were acidified to pH 2.5 with Trifluoroacetic acid (TFA), loaded onto Empore-C18 StageTips[5], eluted with 80% ACN, 0.5% acetic acid, and stored at -80°C until MS analysis. Phosphopeptides were enriched using Phos-TiO beads (GL Sciences) in the presence of 80% ACN, 6% TFA[6], eluted with 15% ammonium hydroxide, 40% ACN, loaded onto Empore-C18 StageTips[5], eluted with 80% ACN, 0.5% acetic acid, and stored at -80°C until MS analysis. The Phos-TiO flow through was collected and processed as described above performing a second round of Phos-TiO enrichment, which was run separately at the MS.

Mass spectrometry analysis

Digested peptides were separated by nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) using a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75–360 µm inner-outer diameter fused silica emitter (New Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5 % acetic acid and buffer B of 0.5 % acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nl/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17-22 min) and eluted by increasing buffer B from 5-15% (22-87 min) and 15-38% (87-147 min), followed by a 10 minute wash to 90% and a 5 min regeneration to 5%. For proteome: survey scans of peptide precursors from 400 to 1500 m/z were performed at 240K resolution (at 200 m/z) with a 1.5×10^5 ion count target and maximum injection time of 50 ms. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 1.6 *m*/*z*, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS^2 ion count target was set to 1×10^4 and maximum injection time of 35 ms. The instrument was run in top speed mode with 3 s cycles. For phosphoproteome: survey scans of peptide precursors from 400 to 1500 *m*/*z* were acquired in the Orbitrap analyzer at 240K resolution (at 200 *m*/*z*) with 2×10^5 ion count target and maximum injection time of 50 ms. Tandem mass spectrometry was performed on the 10 most intense ions by isolation with the quadrupole with isolation window 1.6 *m*/*z*, HCD fragmentation with normalized collision energy of 30, and analysis in the Orbitrap with a resolution of 30K (at 200 *m/z*). The MS^2 ion count target was set to 5×10^4 and maximum injection time of 60 ms. Only those precursors with charge state 2–7 were sampled for MS^2. The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. All data were acquired with Xcalibur software.

Mass spectrometry data analysis and processing

For the thrombin/PAR1 inhibitor dataset: The RAW MS files were processed with MaxQuant computational platform (version 1.5.2.8).[7] Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot[8] (release 2015-02). For the tethered ligand peptides dataset: The RAW MS files were processed with MaxQuant computational platform (version 1.5.3.30).[7] Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot[8] (release 2017-03). Precursor and fragment ions were selected using standard settings. Trypsin with full enzyme specificity and only peptides with a minimum length of 7 amino acids were selected. A maximum of two missed cleavages was allowed. Carbamidomethylation (Cys) was set as fixed modification, while Oxidation (Met), N-acetylation and phospho(STY) as variable modifications. For protein, peptide and phosphorylation site identification, we required a maximum false discovery rate (FDR) of 1%. The relative quantification based on SILAC was performed by MaxQuant enabling the ‘re-quantify’ and ‘match between runs’ options. MS data processing was performed using Perseus version 1.5.5.1 as described before.[3] Briefly, first, reverse and contaminant hits were removed from the MaxQuant

output files. Next, only class I phosphorylation sites, which refer to phosphorylation sites where the phosphorylation could be assigned with a localization probability of at least 0.75 and a score difference ≥ 5[9], were used for the analysis. For each experiment, the normalized SILAC ratios were transformed using the binary logarithm (log_2). For each phosphorylation site, the SILAC ratios were calculated separately for singly, doubly or multiply phosphorylated peptides. Phosphosites were compared with the PhosphoSitePlus database[10] (release 20 March 2017) to determine not previously reported phosphosites. For the thrombin/PAR1 inhibitor dataset: MS data was combined from 3 (10 nM thrombin), 2 (thrombin-TL peptide, vorapaxar + 10 nM thrombin and parmodulin-2 + 10 nM thrombin) or 1 (50 pM thrombin) biological replicate(s), resulting in the following replicates per time point: 7 (10 nM thrombin), 4 (thrombin-TL peptide and parmodulin-2 + 10 nM thrombin), 6 (vorapaxar + 10 nM thrombin), or 3 (50 pM thrombin). Quantified phosphosites were defined as phosphosites where a SILAC ratio was accurately measured in the following number of replicates at each time point: 5 out of 7 (10 nM thrombin), 3 out of 4 (thrombin-TL peptide and parmodulin-2 + 10 nM thrombin), 4 out of 6 (vorapaxar + 10 nM thrombin) or 3 out of 3 (50 pM thrombin). Regulated phosphosites were defined as phosphosites where the SILAC ratio (log_2) was higher than 0.6 in the following number of replicates per time point: 5 out of 7 (10 nM thrombin), 3 out of 4 (thrombin-TL peptide and parmodulin-2 + 10 nM thrombin), 4 out of 6 (vorapaxar + 10 nM thrombin) or 3 out of 3 (50 pM thrombin). For the tethered ligand peptides dataset: MS data was combined from 2 biological replicates, resulting in two replicates per time point per stimulation condition. Quantified phosphosites were defined as phosphosites where a SILAC ratio was accurately measured in both replicates at each time point for each stimulation condition. Regulated phosphosites were defined as phosphosites where the SILAC ratio (log_2) was higher than 0.7 in both replicates of a time point for one of the five stimulation conditions. Interaction network of the regulated phosphosites was made using the Phosphopath plug-in[11] from Cytoscape.[12] The .raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange Consortium http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository[13] with the dataset identifier PXD004264.

Supplementary References

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