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Bioinformatic discovery of novel bioactive peptides

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Short synthetic oligopeptides based on regions of human proteins that encompass functional motifs are versatile reagents for understanding protein signaling and interactions. They can either mimic or inhibit the parent protein's activity[1–4] and have been used in drug development[5]. Peptide studies typically either derive peptides from a single identified protein or (at the other extreme) screen random combinatorial peptides[4,6], often without knowledge of the signaling pathways targeted. Our objective was to determine whether rational bioinformatic design of oligopeptides specifically targeted to potentially signaling-rich juxtamembrane regions could identify modulators of human platelet function. High-throughput *in vitro* platelet function assays of palmitylated cell-permeable oligopeptides corresponding to these regions identified many agonists and antagonists of platelet function. Many bioactive peptides were from adhesion molecules, including a specific CD226-derived inhibitor of inside-out platelet signaling. Systematic screens of this nature are highly efficient tools for discovering short signaling motifs in molecular signaling pathways.

From a set of 2,900 platelet expressed proteins[7], 47 candidate highly expressed transmembrane proteins were identified. To identify functional biomimetic peptides, we enriched for activity by choosing cytoplasmic decamers (<30 residues from membrane) conserved in orthologous sequences of other vertebrates and enriched for molecular diversity by adding peptides from the same region of related human proteins ("paralogous peptides"), in which at least one conserved residue differed markedly[8] (**Supplementary Figs. 1** and **2** online.). We synthesized decamer oligopeptides by standard Fmoc chemistry with N-terminal palmitylation (Pal) (to direct peptides toward the cell membrane[1–3,9]) and desalted and purified by reverse phase HPLC[3,9–11].

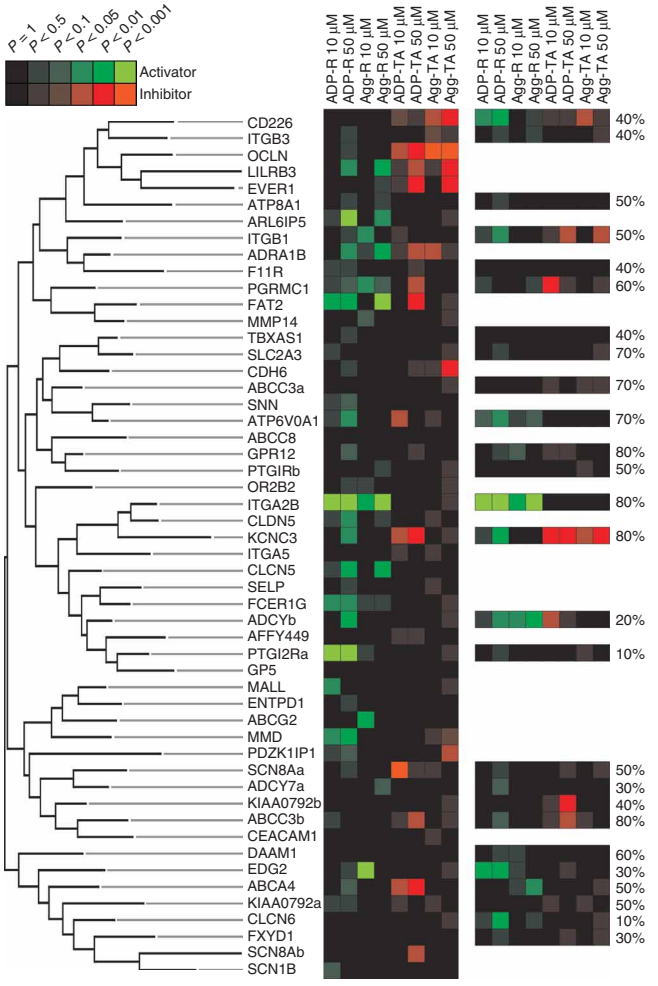

Figure 1 Visualization of significant agonists and antagonists of platelet activation for 52 peptides from 47 platelet proteins. Agonists are green and antagonists are red. Results are for eight assays: ADP release (ADP) and aggregation (Agg) for resting (R) and thrombin-activated (TA) platelets, with two doses of peptide (10 µM and 50 µM). Color intensity is proportional to statistical significance. Right panel: results for 26 paralogous peptides from related human proteins, shown alongside their related platelet protein peptide. Clustering of peptides (to the left) was based on neighbor-joining analysis of ten amino acid physicochemical properties over all residues of each peptide; this particular clustering was chosen from several alternative approaches as the one with the greatest tendency to cluster peptides of similar activity.

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Table 1 Significant effects on platelet function of water- or methanol-soluble peptides

Protein[1]	Type	Peptide	Sequence[2]	Assay[3]	*P* value[4]	Effect[5]	s.e.m.[6]	Activity
ARL6IP5	Glutamate transport	ARL6PI5_Q	NKDVLRRMKK	ADP-R	0.0005	76.3%	9.08%	Agonist
PVRL3	Cell adhesion	CD226_P	RTFRGDYFAK	ADP-R	0.0038	58.7%	6.64%	Agonist
CLCN7	Chloride channel	CLCN6_P	KGNIDKFTEK	ADP-R	0.0073	55.2%	8.13%	Agonist
EDG7	Lysophosphatidic acid receptor	EDG2_P	SISRRRTPMK	ADP-R	0.0045	55.27%	3.12%	Agonist
ITGA2B	Integrin: adhesion	ITGA2B_Q	GFFKRNRPPL	ADP-R	0.0001	89.4%	8.89%	Agonist
ITGAV	Integrin	ITGA2B_P	GFFKRVRPPQ	ADP-R	0.0003	79.1%	5.93%	Agonist
KCNC1	Potassium channel	KCNC3_P	KKHIPRPPQL	ADP-R	0.0040	60.3%	7.08%	Agonist
MMD	Receptor	MMD_Q	WKYLYRSPTD	ADP-R	0.0048	57.4%	6.53%	Agonist
PTGIR	Prostaglandin receptor	PTGIRa_Q	KAVFQRLKLW	ADP-R	0.0004	80.1%	8.95%	Agonist
ADCY4	Adenylate cyclase	ADCY7b_P	EALSSLHSRR	Agg-R	0.0086	8.26%	3.40%	Agonist
ADRA1B	Adrenergic receptor	ADRA1B_Q	SKEFKRAFVR	Agg-R	0.0021	15.9%	4.91%	Agonist
ITGA2B	Integrin: adhesion	ITGA2B_Q	GFFKRNRPPL	Agg-R	0.0008	16.2%	3.88%	Agonist
ITGAV	Integrin	ITGA2B_P	GFFKRVRPPQ	Agg-R	0.0005	17.1%	3.19%	Agonist
LILRB3	Immunoglobulin-like receptor	LILRB3_Q	RRQRHSKHRT	Agg-R	0.0013	13.8%	2.99%	Agonist
ABCA4	ATP-binding cassette receptor	ABCA4_Q	QKKAYTSPEN	ADP-TA	0.0022	34.4%	4.11%	Antagonist
EVER1	Unknown	EVER1_Q	EKKLKRRRKP	ADP-TA	0.0025	39.2%	8.35%	Antagonist
KCNC3	Potassium channel	KCNC3_Q	NKHIPRPPQP	ADP-TA	0.0037	41.4%	13.47%	Antagonist
KCNC1	Potassium channel	KCNC3_P	KKHIPRPPQL	ADP-TA	0.0077	28.1%	5.51%	Antagonist
TMEM63B	Unknown	KIAA0792b_P	TSKMRYKEDD	ADP-TA	0.0037	31.3%	4.33%	Antagonist
OCLN	Tight junction regulation	OCLN_Q	KTRRKMDRYD	ADP-TA	0.0076	30.5%	7.48%	Antagonist
CD226	Adhesion	CD226_Q	RRERRDLFTE	Agg-TA	0.0022	64.9%	9.95%	Antagonist
CDH6	Cell adhesion	CDH6_Q	KKEPLIISKE	Agg-TA	0.0014	65.4%	7.20%	Antagonist
EVER1	Unknown	EVER1_Q	EKKLKRRRKP	Agg-TA	0.0071	53.9%	8.00%	Antagonist
KCNC1	Potassium channel	KCNC3_P	KKHIPRPPQL	Agg-TA	0.0033	68.6%	10.86%	Antagonist
LILRB3	Immunoglobulin-like receptor	LILRB3_Q	RRQRHSKHRT	Agg-TA	0.0056	54.7%	7.21%	Antagonist
OCLN	Tight junction regulation	OCLN_Q	KTRRKMDRYD	Agg-TA	0.0003	79.3%	4.62%	Antagonist

[1]Protein identifiers from approved gene symbol from the HUGO Gene Nomenclature Committee. [2]All peptides have N-terminal palmitylation. Blue indicates positively charged residues. Red indicates negatively charged residues. [3]ADP-R: ADP release from resting platelets induced by peptide. ADP-TA: inhibition by peptide of ADP release from thrombin-activated platelets. Agg-R: aggregation of resting platelets induced by peptide. Agg-TA: inhibition by peptide of aggregation of thrombin-activated platelets. [4]*P* value from two-tailed Mann-Whitney 'U' test. [5]Effect indicates the mean percentage of platelet activation or percentage of inhibition of thrombin-induced activation for the peptide across donors. [6]s.e.m. of percentage of activation. All data is for 50 μM peptides; $P \leq 0.01$.

We experimentally screened 52 peptides (derived from 47 platelet proteins) and 26 paralogous peptides (at 10 μm and 50 μm) in novel 96-well assays for platelet aggregation[12] and ADP secretion[13]. These assays are sensitive to changes in diverse signaling pathways; *in vitro* assays of platelets from human donors have a functional correlate with bleeding and thrombotic disorders, and thus have close relevance to human biology. Moreover, the anucleate platelet favors peptide, rather than RNA, approaches. Agonists of platelet activation were defined as peptides that cause resting platelets to aggregate or secrete ADP, whereas antagonists were defined as peptides that inhibit thrombin-induced platelet aggregation or ADP release.

The screen revealed a large number of active peptides (22 with $P < 0.01$ in one of the four assays for the 50 μM dose; null expectation of three findings), with different peptides typically showing assay-specific effects (**Fig. 1**, **Table 1** and **Supplementary Table 1** online). 13 of the 22 significant peptides showed the same significant effect ($P < 0.05$) at 10 μM, which is consistent with specific, rather than general, nonspecific peptide action. One of the peptides, ITGA2B_Q (1022-Pal-GFFKRNRPPL-1031 of integrin α_{IIb}), contains the previously identified GFFKR[3,14] and RPP[15] motifs, which confirms the ability of the screen to identify known motifs. Its paralogue, ITGA2B_P (1019-Pal-GFFKRVRPPQ-1028 of integrin α_V), shares both motifs and therefore it is not surprising that it also showed activity. However, for five of the evolutionarily related pairs of peptides (ABCA4, CLCN6, KIAA0792b, EDG2 and PTGIRa), only one of the pair had significant activity in any assay (50 μM, $P < 0.01$, **Fig. 1**, **Table 1** and **Supplementary Table 1**), which suggests that activity may be specific to the pathway of a particular protein subfamily member. For three other evolutionarily related pairs that showed activity (ADCY7b, CD226 and KCNC3), both peptides were active in at least one assay (50 μM, $P < 0.01$), but the assays and magnitude of activity observed differed in each case between the two peptides, which is consistent with sequence-specific modes of action. The peptides of one pair actually showed substantially different activities relative to one another: CD226_Q potently inhibited platelet activation (showing a dose-dependent response in both standard light aggregometry and platelet spreading (**Supplementary Fig. 3** online)), whereas the evolutionarily related peptide acted as an agonist (**Figs. 1** and **2**). Such diversity provides a natural library of compounds for investigating motif and domain function[16].

The immunoglobulin-like CD226 has been identified as a platelet[17] and endothelial[18] adhesion factor that binds platelets homophilically[17]. Antibodies to CD226 may inhibit or stimulate platelet activation[19,20]. CD226 is associated with integrin clustering in T cells[19]. CD226 colocalizes with integrins in natural killer cells and T lymphocytes[21], and we observed a strong colocalization in platelets (**Fig. 2**). CD226_Q also inhibited integrin-mediated platelet spreading on immobilized fibrinogen, whereas platelet spreading was not inhibited by the nonpalmitylated peptide (**Fig. 2**). Moreover, CD226_Q inhibited platelet responses induced by thrombin, but not direct integrin activation by DTT ($P = 0.86$, in contrast with thrombin, for which $P = 0.000$, **Fig. 2**). Thus, this peptide reveals a role for the CD226 protein in regulation of inside-out but not outside-in integrin activation. Identification of an inhibitor of inside-out signaling in an

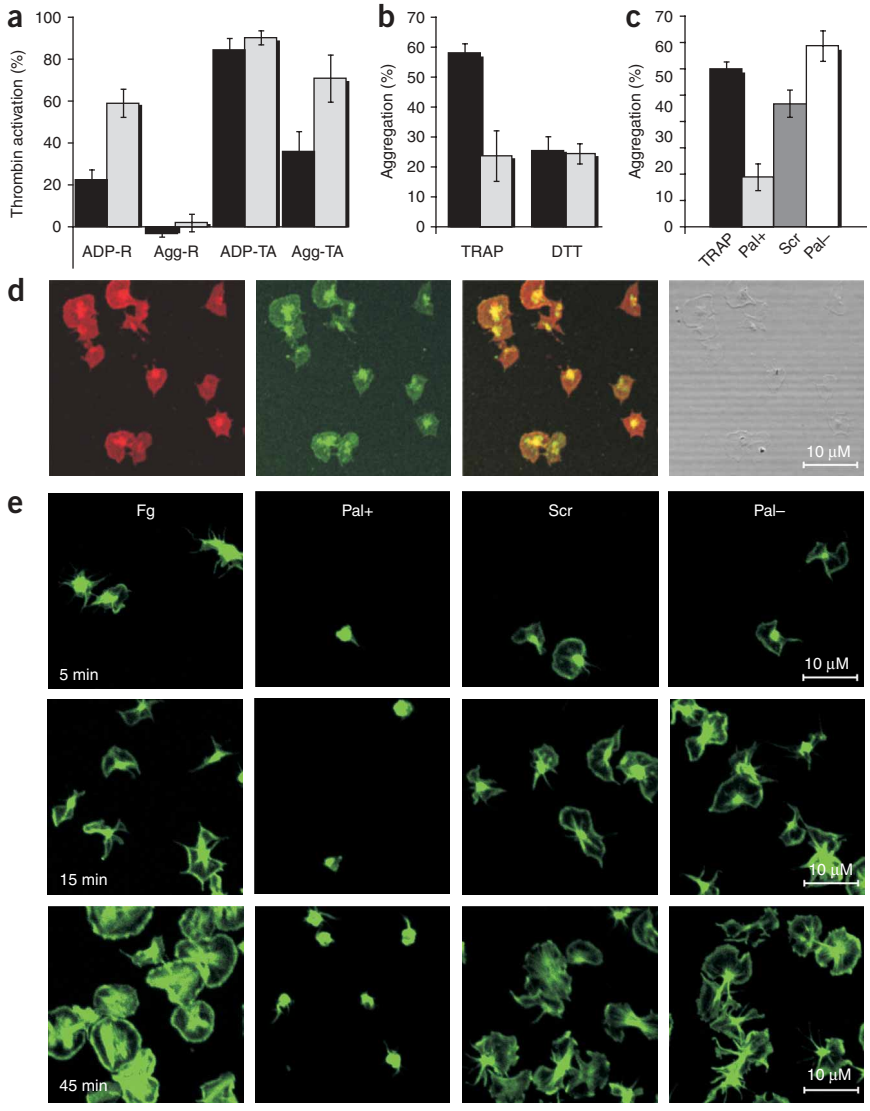

Figure 2 Effect of the CD226_Q peptide, derived from the CD226 protein, on platelet function. (**a**) Results of high-throughput assays for CD226_Q peptide (Pal-RRERRDLFTE; black) and its nonplatelet paralogue CD226_P (Pal-RTFRGDYFAK; light gray). (**b**) Inhibition of standard platelet aggregation by CD226_Q peptide. Platelets were preincubated with buffer (black) or peptide (light gray; 50 μM) for 3 min before the addition of 10 μM thrombin receptor activation peptide (TRAP) or 5 mM DTT in the presence of 1 mg ml^{-1} fibrinogen. (**c**) Inhibition of TRAP-induced platelet aggregation by CD226_Q peptide variants (50 μM): TRAP, TRAP alone; Pal+, palmitylated peptide Pal-RRERRDLFTE; Scr, C-terminal scrambled peptide Pal-RRERRETFDL; Pal–, nonpalmitylated peptide RRERRDLFTE. (**d**) Colocalization of CD226 protein with the platelet integrin $\alpha_{IIb}\beta_3$ in platelets activated by spreading on immobilized fibrinogen (20 μg ml^{-1}) for 45 min; green, CD226; red, $\alpha_{IIb}\beta_3$; yellow, colocalization. (**e**) Inhibition of platelet spreading on fibrinogen by CD226_Q peptide. Gel-filtered human platelets (3×10^5 μl^{-1}) were allowed to adhere and spread on fibrinogen-coated glass slides (20 μg ml^{-1}) for the indicated times. Platelets were left untreated (Fg: fibrinogen alone) or preincubated for 3 min with 50 μM peptide. Platelets are stained for polymerized actin with fluorescein isothiocyanate phalloidin. Error bars are s.e.m.

integrin-associated protein represents a new potential target mechanism for antiplatelet therapy. Three paralogous peptides (from EDG7, CLCN7 and TMEM63B) showed pronounced effects on platelet signaling when the queries did not (**Table 1**). All three are actually derived from proteins that are also expressed in platelets[22].

To investigate whether bioactivity is caused by nonspecific features of the peptides, we property-clustered peptides; but we did not find very strong trends relating properties and activity (**Fig. 1** and **Supplementary Methods** online). The most suggestive clustering, based on position-independent physicochemical properties, clustered four peptides (CD226_Q, EVER1_Q, LILRB3_Q and OCLN_Q) that all inhibited platelet aggregation (**Fig. 1**). However, the clustering was relatively weak, with no single common feature uniquely defining these four peptides (see charge distributions, **Table 1**). Two other aggregation-inhibiting peptides (CDH6_Q and KCNC3_P) did not group with this cluster, and the three other assays did not form obvious clusters (**Fig. 1**), which suggests that the weak clustering observed may have been a chance finding. Scrambling of peptide sequences can assess the influence of residue composition. We investigated scrambled peptides for two of the most active property-clustered aggregation antagonists, CD226_Q (280-Pal-RRERRDLFTE-289, derived from CD226) and OCLN_Q (266-Pal-KTRRKMDRYD-275, derived from OCLN), which also share a positively charged N terminus. Because short motifs easily arise by chance in randomized sequences from compositionally homogeneous peptides (such as the positively charged CD226_Q and OCLN_Q), scrambling the entire sequence is not an effective means of determining sequence specificity. Therefore, we scrambled the less charged C termini to test whether these more heterogeneous regions of the peptides determine specificity. The C-terminally scrambled Pal-RRERRETFDL peptide of CD226_Q lacked the strong aggregation-antagonist effect of the original peptide (**Fig. 2c**,**e**), whose action therefore seems to be dependent on the more unique C terminus. In contrast, the OCLN_Q peptide and its C-terminally scrambled Pal-KTRRKKDYRDM peptide showed a similarly strong aggregation antagonism (data not shown), which suggests that their activity may reflect their N-terminal charge distribution. Thus, the set of bioactive peptides includes motifs with varying degrees of specificity. This is to be expected, given the varying information content of motifs already known[23]. Finally, we tested nonpalmitylated forms of CD226_Q and OCLN_Q, and, as previously demonstrated for other peptides[24,25], neither was active in its nonpalmitylated form (**Fig. 2**).

Given the evidence that the activities observed are due to specific sequence features, we sought to identify possible shared motifs in the peptides that might account for similar activities in different peptides (correcting for evolutionary relationships between some peptide pairs)[26] (**Supplementary Methods**). Shared motifs were not strongly enriched among the active peptides (**Supplementary Table 2** online). Only 2 of the 20 top-ranked motifs identified among the 78 peptides,

KXXYXSP and RPPQ, were shared by active peptides, which is consistent with a random distribution among active and inactive peptides. The RPPQ motif is found in peptides derived from KCNC3, KCNC1 and ITAV. Given that RPP is implicated in energy-dependent integrin signaling[15], potassium channel and integrin peptides (**Table 1**) could potentially act through a common broad mechanism—but clearly not an identical mechanism, as ITGA2B_Q is an agonist and KCNC3_Q is an antagonist. The KXXYXSP motif is also found in peptides with opposite activities (such as the activator MMD_Q and the inhibitor ABCA4_Q; **Table 1**), which indicates that, even if they too act through a common partner, there is additional specificity in the rest of their sequences. Notably, the most similarity between any two unrelated peptides in the study was an RDLFT motif shared by CD226_Q and ADCY7a_Q, which includes four of five C-terminal residues of CD226_Q that (as indicated by scrambling) are strongly implicated in the inhibitory activity of this peptide (**Fig. 2**). ADCY7a_Q, however, showed no significant activity, which suggests that despite its obvious importance, the C terminus alone is not sufficient for activity.

Could these peptides have been identified by other computational approaches? We sought to identify (i) additional known motifs in the sequences, and (ii) novel motifs shared among proteins that share a protein-protein interaction partner. Only two known "ligand binding" motifs from the Eukaryotic Linear Motif database[23] occurred (with expectation <1) in the 78 peptides (**Supplementary Methods**). Notably, one of these—the SH3_1 binding motif in the peptides from KCNC3 and KCNC1—recalls the SH3_1 binding of other potassium channels via similar proline-rich motifs[27]. Overall, though, it seems that most of the active peptides do not work through previously known and easily identified motifs. Computational discovery of motifs among related proteins that share an interaction partner is an alternative strategy for motif characterization[28]. A screen for motifs shared among proteins that interact with a common partner suggested four possible interaction motifs in active peptides (**Supplementary Table 3** online) but did not identify motifs for most of the active peptides. Therefore, our approach complements the approach of discovering motifs shared by common interaction partners[28] because it can define bioactive motifs that lack convergently evolved instances in proteins of known related function.

We conclude that systematic biomimetic oligopeptide screens are a highly efficient tool for discovering short signaling motifs in molecular signaling pathways; discovered peptides represent potential molecular templates for drug development. Hundreds of such short signaling motifs await discovery in the human proteome[28]. It is encouraging that our screen is enriched for adhesion protein–derived peptides (cadherins, occludin, integrins, LILRB3 and CD226); identifying and modeling the action of short interaction motifs represents a key strategy in designing small molecules that target adhesion in a wide range of therapeutic areas.

METHODS

Peptide design. The objective was to identify peptides that span residues that are strongly similar (conserved) to those in the corresponding proteins in other species (orthologs), but that differ from those in related human proteins (paralogues) (**Supplementary Fig. 1**). The peptides also had to lie within 30 amino acids of the membrane. Ancestral sequence predictions from HAQESAC (http://bioinformatics.ucd.ie/shields/software/haqesac/index.html) were used to calculate "burst after duplication" (BAD) statistics for each residue, which scores the number of amino acid property differences between the common ancestor of the paralogues and the common ancestor of the platelet query protein's orthologs[29]. BAD is built on the underlying assumption that sites critical to changes in gene function between paralogues are marked by a burst of radical amino acid substitutions directly after duplication, which are subsequently conserved within orthologous groups. Residues that are conserved within subfamilies but differ between them receive a high score and identify potential functional diversity in the corresponding regions of the parent proteins. (**Supplementary Fig. 2** and **Supplementary Methods**). For query proteins with human paralogues, we designed decamer peptides that span membrane-proximal cytoplasmic residues in well-aligned regions with high BAD scores. For queries without paralogues, decamer peptides were selected on the basis of evolutionary conservation. We avoided unfavorable amino acid combinations and excessively hydrophobic peptides to reduce the risk of by-products during synthesis, postsynthetic degradation, and solubility problems. Further details on peptide design are available in **Supplementary Methods**.

Peptide synthesis. See **Supplementary Methods**.

Rationale for platelet assays. See **Supplementary Methods**.

High-throughput platelet aggregation assays. We assayed each peptide at two doses (10 μM and 50 μM) on six donors (three male and three female)[12] and expressed platelet activation as a proportion of the maximum activation observed for thrombin in the presence of the peptide vehicle on its own. Washed platelets were prepared as described previously[3] and diluted in buffer A (130 mM NaCl, 10 mM trisodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4) to a concentration of 6×10^8 ml^{-1}. Peptide stock solutions (1 mM) were prepared in an appropriate vehicle depending on individual solubility (H_2O, DMSO, 1% w/v; or methanol, 5% w/v final concentration; see **Supplementary Table 1**) and stored at –80 °C. We performed dual agonist-antagonist assessment of platelet aggregation as follows: platelets (80 μl) and peptides (10 μM and 50 μM) or the relevant vehicle were added to a 96-well plate to a final volume of 100 μl and shaken at 37 °C. Absorbance readings (405 nm; 0.1 s; Wallac Victor^2 1420 spectrophotometer) were taken before addition of peptide (T0) and at subsequent 3-min intervals. Thrombin (0.2 units ml^{-1}) was added after 6 min (T6), and an additional two absorbance readings were taken at T9 and T12. Changes in absorbance reflected agonist or antagonist properties of peptides to promote or inhibit platelet aggregation.

High-throughput ADP secretion assays. We diluted platelets prepared as described above to 3×10^8 ml^{-1} and aliquoted into black and white 96-well isoplates. We added peptides (at 10 μM and 50 μM) or the relevant vehicle and shook the plates at 37 °C for 3 min. Chronolume (10 μl) was added after 3 min and luminescence recorded on a Wallac Victor^2 1420 multilabel counter (Perkin Elmer) to measure the peptide-induced ADP release. Parallel plates were prepared with platelets activated by 0.2 units ml^{-1} of thrombin. Changes in absorbance reflected agonist or antagonist properties of peptides to promote or inhibit ADP release.

CD226_Q inside-out versus outside-in signaling. See **Supplementary Methods**.

Confirmatory confocal microscopy. See **Supplementary Methods**.

Platelet spreading and phalloidin staining. See **Supplementary Methods**.

Statistical analysis of peptide activity. To compensate for differences in assay readings for different donors and solvents, we converted raw aggregation and ADP secretion scores into a 'percentage (thrombin-induced) activation' (*%Act*) reading. Mean prethrombin controls were subtracted to give a resting baseline value of zero. Readings were then scaled by mean post-thrombin controls to give thrombin activation a value of 100%:

$$\%Act = 100\% \times (R - C_n)/(C_t - C_n)$$

where R is the raw reading, C_n is the mean vehicle control reading before thrombin has been added, and C_t is the mean vehicle control after thrombin.

We compared *%Act* values for each peptide to all other peptides using a Mann-Whitney nonparametric test. For comparison, peptides were also compared with vehicle controls using a Wilcoxon nonparametric paired *t*-test of raw assay data. Each well for each peptide was individually paired with the mean value for the relevant vehicle controls on the same donor. Comparisons

between peptide and paralogue were performed using the Mann-Whitney 'U' test. Statistical significance of follow-up P-selectin and aggregometry analyses was determined by one-tailed *t*-tests.

Additional methods. See **Supplementary Methods**.

Note: Supplementary information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

D.C.S., D.K. and N.M. devised the experiment; R.J.E. and D.C.S. formulated the bioinformatics peptide design algorithm and performed the statistical analysis; R.J.E., N.M., D.K. and D.C.S. cowrote the paper; R.J.E. developed the bioinformatics tools and performed the peptide design and the bioinformatics searches for shared or known motifs in bioactive peptides; M.D. designed the selection rules for the synthesis of the peptides to ensure maximum integrity, stability and solubility of the corresponding palmitylated sequences and supervised the synthesis of these peptides; N.M., A.K. and E.D. developed and performed the high-throughput platelet activation assays; G.M. performed the colocalization microscopy; W.S., D.K. and N.M. designed and interpreted the platelet spreading assays; S.D.E.P. performed platelet expression analysis; M.F. provided proteomics data.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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