Regulation of the thrombin/protease-activated receptor 1 axis by chemokine (C-X-C motif) receptor 4

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Abstract

The chemokine receptor CXCR4, a G protein-coupled receptor (GPCR) capable of heteromerizing with other GPCRs, is involved in many processes including immune responses, hematopoiesis and organogenesis. Evidence suggests that CXCR4 activation reduces thrombin/protease activated receptor 1 (PAR1)-induced impairment of endothelial barrier function. However, the mechanisms underlying cross-talk between CXCR4 and PAR1 are not well understood. Using intermolecular bioluminescence resonance energy transfer and proximity ligation assays, we found that CXCR4 heteromerizes with PAR1 in the HEK293T expression system and in human primary pulmonary endothelial cells (hPPECs). A peptide analogue of transmembrane domain 2 (TM2) of CXCR4 interfered with PAR1:CXCR4 heteromerization. In HTLA cells, the presence of CXCR4 reduced the efficacy of thrombin to induce β-arrestin-2 recruitment to recombinant PAR1 and enhanced thrombin-induced Ca\(^{2+}\) mobilization. While thrombin-induced extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation occurred more transiently in the presence of CXCR4, peak ERK1/2 phosphorylation was increased when compared with HTLA cells expressing PAR1 alone. CXCR4 associated effects on thrombin-induced β-arrestin-2 recruitment to and signaling of PAR1 could be reversed by TM2. In hPPECs, TM2 inhibited thrombin-induced ERK1/2 phosphorylation and activation of ras homolog gene family member A. CXCR4 siRNA knockdown inhibited thrombin-induced ERK1/2 phosphorylation. While thrombin stimulation reduced surface expression of PAR1, CXCR4 and PAR1:CXCR4 heteromers, chemokine (C-X-C motif) ligand 12 stimulation reduced surface expression of CXCR4 and PAR1:CXCR4 heteromers, but not of PAR1. Finally, TM2 dose-dependently inhibited thrombin-induced impairment of hPPEC monolayer permeability. Our findings suggest that CXCR4:PAR1 heteromerization enhances thrombin-induced G protein signaling of PAR1 and PAR1-mediated endothelial barrier disruption.

Introduction

Chemokine (C-X-C motif) receptor 4 (CXCR4) is essential during embryonic development and plays multi-faceted roles in biology after birth (1-4). CXCR4 is also involved in numerous disease processes and the CXCR4 antagonist AMD3100 has already reached the clinical arena as a drug approved by the Federal Drug Administration for mobilization of stem cells in cancer patients (5-7). Previously, CXCR4 agonists have been reported to reduce inflammation-induced vascular hyper-permeability in animal models and to inhibit impairment of endothelial cell barrier function induced by the thrombin/protease activated receptor 1 (PAR1) axis (8-18). The molecular mechanisms by which CXCR4 regulates the thrombin/PAR1 axis in endothelial cells, however, are not well understood.

The majority of research on the roles of CXCR4 in health and disease has focused on CXCR4-mediated downstream signaling events to understand the mechanisms underlying its pleiotropic functions. Nevertheless, several lines of evidence suggest that the formation of heteromeric complexes with other receptors is another molecular mechanism through which CXCR4 regulates cell function. CXCR4 has been reported to form heteromeric complexes with multiple other G protein-coupled receptors (GPCRs), such as chemokine (C-C motif) receptor 2 (CCR2), CCR5, CXCR3, atypical chemokine receptor (ACKR) 3, chemerin receptor 23, α\(_{1}\)A/B/D-adrenergic receptors (ARs), β\(_2\)-AR, δ-opioid receptor, vasopressin receptor 1A, cannabinoid receptor 2 or the virally-encoded GPCR of Herpesvirus saimiri, leading to altered pharmacological properties of the interacting receptor partners (19-33). In the present study we tested whether heteromerization of CXCR4 with PAR1 could be a molecular mechanism that contributes to the previously observed cross-talk between the receptors. Here we provide evidence suggesting formation of CXCR4:PAR1 heteromers in recombinant systems and in human primary pulmonary endothelial cells (hPPEC), through which CXCR4 regulates PAR1 signaling and function upon thrombin activation.
Results and Discussion

PAR1 heteromerizes with CXCR4

We utilized intermolecular bioluminescence resonance energy transfer (BRET) titration assays to test whether CXCR4 heteromerizes with PAR1 (Fig. 1A). Consistent with non-specific bystander BRET signals, BRET signals in cells transfected with CXCR4-RLuc and YFP were low and increased linearly with increasing energy acceptor:donor ratios. BRET signals in cells transfected with CXCR4-RLuc and PAR1-YFP showed hyperbolic progression with increasing energy acceptor:donor ratios (Fig. 1A). BRET was independent of the concentrations of BRET partners when tested at fixed acceptor:donor ratios (Fig. 1B), suggesting constitutive heteromerization between CXCR4 and PAR1 in a recombinant cell system (34).

To assess whether endogenously expressed CXCR4 and PAR1 heteromerize in hPPECs, we employed proximity ligation assays (PLA) to visualize individual receptors and proximity between CXCR4 and PAR1. To confirm selectivity of anti-PAR1 for its GPCR target (35), we first analyzed staining of hPPECs with anti-PAR1 by flow cytometry and PLA. We observed more than 50% reduction of signals in hPPECs after incubation with PAR1 siRNA, as compared with cells incubated with vehicle. Incubation of cells with a control TM peptide did not significantly affect PLA signals corresponding to individual receptors or CXCR4:PAR1 heteromers in hPPECs. To confirm that the TM2 peptide interferes with CXCR4:PAR1 heteromerization, we transfected HEK293T cells, which endogenously express PAR1 (37,38), with HA-CXCR4 and performed PLA with anti-PAR1 and anti-HA. As shown in Fig. 2C/D, the TM2 peptide did not affect PLA signals for individual receptors, but reduced PLA signals corresponding to HA-CXCR4:PAR1 heteromers by 45 ± 4%.

Because disruption of TM domains of GPCRs with TM-derived peptides can affect receptor heteromerization through interference with the correct assembly of the target membrane protein (29-31,39,40), our findings suggest similarity of the CXCR4 interactions sites for PAR1 and α1A/B-adrenoceptors, which appear to be distinct from the interactions sites of CXCR4 for ACKR3 (28).

The presence of CXCR4 reduces thrombin-induced β-arrestin-2 recruitment to PAR1

Because GPCR heteromerization may lead to signaling complexes with pharmacological properties distinct from the receptor protomers, we tested whether heteromerization of PAR1 with CXCR4 would affect thrombin-induced β-arrestin-2 recruitment to PAR1 utilizing the PRESTO-Tango (parallel receptorome expression and screening via transcriptional output, with transcriptional activation following arrestin-2 translocation) system (41). In this assay, HTLA cells stably expressing a tetracyclin transactivator (tTA)-dependent luciferase reporter and a β-arrestin-2 - Tobacco Etch Virus protease (TEV) fusion gene, are transfected with DNA encoding a GPCR that contains the sequences for a TEV cleavage site followed by tTA at the 3’-end (GPCR-Tango). β-arrestin-2 recruitment upon receptor
activation leads to cleavage of the TEV site and the release of tTA, which results in the transcription of luciferase, thus permitting luminescence measurements upon addition of luciferase substrate. We transfected HTLA cells with PAR1-Tango plus pcDNA3 or CXCR4 and confirmed similar expression of PAR1-Tango and expression of CXCR4 by flow cytometry (Fig. 3A). While the presence of CXCR4 did not affect the potency of thrombin to induce β-arrestin-2 recruitment to PAR1 (EC_{50}: PAR1-Tango/pcDNA3: 153 ± 78 nM; PAR1-Tango/CXCR4: 77 ± 81 nM, p>0.05), the efficacy of thrombin to induce β-arrestin-2 recruitment was significantly reduced by the presence of CXCR4 (Fig. 3 B).

Next, we tested whether the effect of CXCR4 on thrombin-induced β-arrestin-2 recruitment to PAR1 can be reversed by interference with CXCR4:PAR1 heteromerization with the TM2 peptide. The TM2 peptide is known to function as a biased CXCR4 antagonist, which inhibits G protein signaling of CXCR4 but permits β-arrestin-2 recruitment to the receptor (40,42,43). After pre-treatment of cells with the control peptide, the efficacy of thrombin to induce β-arrestin-2 recruitment to PAR1 was significantly reduced in cells co-expressing PAR1-Tango and CXCR4, as compared with cells transfected with PAR1-Tango alone (Fig. 3C). The presence of CXCR4 did not affect the potency of thrombin to induce β-arrestin-2 recruitment to PAR1 in cells pretreated with the control peptide (EC_{50}: PAR1-Tango/pcDNA3: 52 ± 8 nM; PAR1-Tango/CXCR4: 81 ± 14 nM, p>0.05). These findings reproduce our observations in cells not exposed to the control peptide (Fig. 3B). While pre-treatment of cells with the TM2 peptide did not affect thrombin-induced β-arrestin-2 recruitment to PAR1 in cells expressing PAR1-Tango alone, it restored the efficacy of thrombin to induce β-arrestin-2 recruitment to PAR1 in cells co-expressing PAR1-Tango and CXCR4. These findings suggest that CXCR4 within CXCR4:PAR1 heteromers reduces β-arrestin-2 recruitment to PAR1, and that the TM2 peptide abolishes this effect via interference with CXCR4:PAR1 heteromerization.

The presence of CXCR4 enhances thrombin-induced G protein signaling of PAR1

To test whether heteromerization of PAR1 with CXCR4 also modulates thrombin-induced G protein signaling of PAR1, we utilized intracellular Ca^{2+}-fluxes and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Thr202/Tyr204) as read-outs. We first confirmed that the TM2 peptide does not interfere with intracellular Ca^{2+} mobilization upon thrombin stimulation in HTLA cells expressing PAR1-Tango (Fig. 3D). We then compared maximal thrombin-induced Ca^{2+} fluxes in HTLA cells expressing PAR1-Tango with or without CXCR4 after pre-treatment with the TM2 or the control peptide (Fig. 3E). Ca^{2+} fluxes in HTLA cells expressing PAR1-Tango alone were indistinguishable in cells pre-treated with the TM2 or control peptide. In combination with our observation that the TM2 peptide does not affect Ca^{2+} fluxes in HTLA cells expressing PAR1-Tango, when compared with vehicle treated cells (Fig. 3D), these data indicate that both peptides do not interfere directly with PAR1 signaling.

The presence of CXCR4 significantly increased maximal Ca^{2+} responses in cells pre-treated with the control peptide, but not in cells pre-treated with the TM2 peptide (Fig. 3E). Similarly, thrombin-induced ERK1/2 phosphorylation in HTLA cells expressing PAR1-Tango alone was indistinguishable between cells pre-treated with the TM2 or control peptide (Fig. 4A/B). The presence of CXCR4, however, increased peak ERK1/2 phosphorylation in cells pre-treated with the control peptide, but not in cells pre-treated with the TM2 peptide (Fig. 4C/D). These observations imply that CXCR4 within CXCR4:PAR1 heteromers enhances G protein signaling of thrombin-activated PAR1, which can be prevented by disrupting PAR1:CXCR4 heteromers with the TM2 peptide. While G protein-mediated ERK1/2 phosphorylation has been shown to occur rapidly and transiently, β-arrestin-mediated ERK1/2 phosphorylation occurs delayed and is sustained over longer time periods (44,45). As shown in Fig. 4D, we observed that thrombin-induced ERK1/2 phosphorylation in cells co-expressing CXCR4 and PAR1 was significantly reduced at later time points after pre-treatment with the TM control peptide, as compared with cells pre-treated with the TM2 peptide. In combination with the reduced efficacy of thrombin to recruit β-arrestin-2 to PAR1 in the presence of CXCR4, our observations point toward
reduced thrombin-induced β-arrestin-2-mediated signaling from the CXCR4:PAR1 heteromer.

**Interference with CXCR4:PAR1 heteromerization inhibits thrombin-induced signaling in human primary pulmonary endothelial cells**

To assess whether the TM2 peptide modulates thrombin-induced signaling in hPPECs, we measured ERK1/2 phosphorylation by Western blotting and Ras homolog gene family member A (RhoA) activation utilizing Rho-GTP pull-down assays (46,47). Fig. 5A shows representative images from Western blotting experiments for the detection of pERK1/2 and total ERK in cell lysates from hPPECs and Fig. 5B the densitometric quantification of ERK1/2 phosphorylation from 5 independent experiments. Treatment of hPPECs with the TM2 peptide and the control peptide did not affect ERK1/2 phosphorylation. While thrombin stimulation increased ERK1/2 phosphorylation in cells pre-treated with vehicle and the TM control peptide, thrombin-induced ERK1/2 phosphorylation was inhibited in cells pre-treated with the TM2 peptide. Because these data suggest that disruption of endogenously expressed CXCR4:PAR1 heteromers inhibits thrombin-induced ERK1/2 phosphorylation, we tested whether depletion of CXCR4 from the cell surface of hPPECs by RNA interference would also affect thrombin-induced signaling. We utilized flow cytometry to confirm knockdown of CXCR4 and to document unchanged expression of PAR1 in cells incubated with CXCR4 siRNA, when compared with cells incubated with non-targeting siRNA (Fig. 5C). Fig. 5D shows a representative image from Western blotting experiments for the detection of pERK1/2 and total ERK in cell lysates from hPPECs after incubation with NT or CXCR4 siRNA and Fig. 5E the densitometric quantification of ERK1/2 phosphorylation from 3 independent experiments. Consistent with our observations on the effects of the TM2 peptide, depletion of CXCR4 from the cell surface of hPPECs significantly reduced thrombin-induced ERK1/2 phosphorylation.

Fig 5F shows representative images from Western blots after RhoA-GTP pull-down from hPPEC lysates and Fig. 5G the densitometric quantification of the relative amounts of GTP-bound RhoA from 3 independent experiments. Pre-treatment of cells with the TM2 peptide or with chemokine (C-X-C motif) ligand 12 (CXCL12) for 30 min did not affect the RhoA-GTP/total RhoA ratios in cell lysates. The latter is consistent with the rapid and transient nature of CXCR4-mediated RhoA activation (48). While the RhoA-GTP/total RhoA ratio was increased in hPPECs 5 min after thrombin stimulation, this effect was inhibited in cells pre-treated with the TM2 peptide or CXCL12. Collectively, our observations in hPPECs suggest that interference with PAR1:CXCR4 heteromerization inhibits thrombin-induced G protein mediated signaling of PAR1, which is consistent with our findings in expression systems. CXCL12 has previously been reported to enhance normal endothelial cell barrier function through CXCR4-mediated activation of the phosphoinositide 3-kinase / Ras-related C3 botulinum toxin substrate 1 (Rac1) pathway (9). The signaling events through which CXCL12 antagonizes thrombin-induced impairment of endothelial cell barrier function, however, are unclear. Because RhoA activation is a signaling event critical to PAR1-mediated endothelial barrier disruption upon thrombin stimulation (49-51), the inhibitory effect of CXCL12 on thrombin-induced RhoA activation that we observed implies that inhibition of thrombin-induced RhoA activation contributes to protective effects of various natural and synthetic CXCR4 agonists that have been observed previously (8,9,12). Inhibition of ERK1/2 phosphorylation and of RhoA activation has recently been described for the agonist-bound heterodimer between CXCR4 and cannabinoid receptor 2 (26,48). Therefore, it appears possible that the CXCR4:PAR1 heterodimer exhibits a similar pharmacological behavior.

**CXCL12 stimulation reduces expression of CXCR4 and CXCR4:PAR1 heteromers, but does not affect PAR1 expression**

While CXCR4 is known to internalize upon CXCL12 binding in a β-arrestin-mediated mechanism, we provided evidence that the ACKR3:AVPR1A and CXCR4:α1B-AR heteromers internalize upon binding to only one of the agonists (30,31,52,53). Thus, as an alternative explanation for the effects of CXCL12 on thrombin-induced RhoA activation and endothelial
cell barrier function, depletion of the CXCR4:PAR1 heteromer from the cell surface upon binding of CXCL12 to CXCR4 could contribute to these effects. To test this possibility, we pre-treated hPPECs with the TM2 or the TM control peptide, followed by stimulation with thrombin or CXCL12. Quantification of CXCR4 and PAR1 expression by flow cytometry (Fig. 6A-C) and by PLA (Fig. 7A-D) demonstrated consistently that agonist-stimulation of hPPECs reduced expression of the corresponding receptors. This effect was not affected by the presence of the TM peptides. Accordingly, quantification of PLA signals showed reduction of CXCR4:PAR1 heteromer levels after stimulation with both agonists (Fig. 7E). While CXCL12 stimulation did not affect PAR1 expression (Fig 6C and 7D), thrombin stimulation reduced expression of CXCR4 in cells pre-treated with the TM control peptide, but not in cells pre-treated with the TM2 peptide (Fig. 6B and 7C). These data suggest that thrombin activation of PAR1 within CXCR4:PAR1 heteromers results in co-internalization of both receptors, whereas CXCL12 binding to CXCR4 within CXCR4:PAR1 heteromers selectively depletes CXCR4 from the cell surface. Because PAR1 is known to internalize via a dynamin- and clathrin-dependent pathway that is independent of β-arrestins (51,54), differences between the mechanisms that regulate CXCR4 and PAR1 internalization likely account for such asymmetrical agonist-induced effects on the CXCR4:PAR1 heteromer. It is of note that asymmetrical agonist and antagonist-induced effects have previously been described for other GPCR heteromers (30,31,55,56). Our observation that CXCL12-induced CXCR4 internalization reduces expression of CXCR4:PAR1 heteromers without affecting PAR1 expression suggests that CXCL12 stimulation increases the proportion of PAR1 that is not in contact with CXCR4, which will enhance β-arrestin-2 recruitment to PAR1 and inhibit G protein-mediated signaling of PAR1 upon thrombin stimulation.

**Interference with CXCR4:PAR1 heteromerization inhibits thrombin-induced disruption of endothelial barrier function**

Because the TM2 peptide inhibited thrombin-induced ERK1/2 phosphorylation and RhoA activation in hPPECs, we tested whether these effects on intracellular signaling events correspond to functionally relevant effects on endothelial cell barrier function. While the TM2 peptide (50 µM) did not affect hPPEC monolayer permeability for FITC-dextran, thrombin-induced hPPEC monolayer hyperpermeability could be inhibited with the TM2 peptide in a dose-dependent manner (Fig. 8A). The control TM peptide did not affect thrombin-induced hyperpermeability (Fig. 8B).

The apparent paradox that the CXCR4 agonist CXCL12 as well as the TM2 peptide, which inhibits G protein-mediated signaling and function of CXCR4, inhibit thrombin-induced G protein mediated signaling of PAR1 and endothelial barrier function impairment can be explained by the similarity of their effects on CXCR4:PAR1 heteromerization. Both molecules reduce expression of CXCR4:PAR1 heteromers on the cell surface without affecting PAR1 expression levels. Whereas thrombin-induced PAR1 activation is known to impair endothelial barrier function predominantly via G protein-mediated signaling, such as ERK1/2 and RhoA activation, occupancy of endothelial protein C receptor by activated protein C (APC) has been shown to bias PAR1 signaling upon APC and thrombin activation towards β-arrestin-2 mediated cytoprotective signaling (50,51,57). Our findings suggest that CXCR4 within CXCR4:PAR1 heteromers enhances thrombin-induced G protein-mediated signaling of PAR1 and facilitates impairment of endothelial barrier function, which can be prevented by interference with CXCR4:PAR1 heteromerization.

The possible roles of β-arrestins in this process, however, are currently difficult to assess because β-arrestin-1 and -2 fulfill distinct roles in regulating PAR1 signaling and our observations are limited to β-arrestin-2. Unlike β-arrestin-1, β-arrestin-2 has been reported not to play a significant role in PAR1 uncoupling from G protein signaling (54,58). In contrast, cytoprotective β-arrestin-mediated signaling of PAR1 has been attributed to β-arrestin-2 (50,51,57). Our observations on the effects of CXCR4 on thrombin-induced β-arrestin-2 recruitment to PAR1 and on the temporal pattern of thrombin-induced ERK1/2 phosphorylation are consistent with reduced β-arrestin-2 mediated signaling from the CXCR4:PAR1 heteromer.
Thus, CXCR4 within CXCR4:PAR1 heteromers may bias PAR1 signaling by enhancing G protein and reducing β-arrestin-2 signaling. Because thrombin, however, is known to recruit β-arrestin-1 and -2 to PAR1, it appears possible that the efficacy of thrombin to recruit β-arrestin-1 to PAR1 within CXCR4:PAR1 heteromers is also reduced, which would imply reduced PAR1 uncoupling of G protein signaling as a mechanism by which CXCR4 enhances G protein signaling. Detailed studies in the functional roles of β-arrestins in thrombin-induced signaling from the CXCR4:PAR1 heteromer will be required to answer these questions in the future.

Conclusively, the findings of the present study identify PAR1 as another GPCR heteromerization partner of CXCR4 and provide evidence for pharmacologically relevant functions of the CXCR4:PAR1 heteromer. Our findings suggest a molecular mechanism that likely contributes to the previously described protective effects of CXCR4 agonists on thrombin-induced endothelial barrier function impairment in vitro and on inflammation-induced vascular hyperpermeability in preclinical disease models. Our observations further support the concept that the development of drugs targeting GPCR heteromers could provide new therapeutic opportunities in the future.

**Experimental procedures**

**Proteins, peptides and reagents**

CXCL12 was purchased from Protein Foundry (Milwaukee, Wisconsin) and human alpha thrombin from Enzyme Research Laboratories (South Bend, IN). The peptide analogue of transmembrane helix 2 (TM2) of CXCR4 was as described (28,31,42,43,59). A peptide analogue of TM4 of α1A-AR (IVNLAVADLLLTSTVLPFSAIFEVDDD) was used as a control peptide. Solid-phase synthesis on a 433A Applied Biosystems Peptide Synthesizer using Fmoc amino acid derivatives was used for the production of both peptides. After cleavage with 87.5% (vol/vol) trifluoroacetic acid containing 5% (vol/vol) water, 5% (vol/vol) thioanisol, and 2.5% (vol/vol) triisopropylsilane, the peptides were purified by reverse phase HPLC using an Atlantis C3 column (Agilent Technologies). The peptide structure and purity was confirmed by ion-spray mass-spectrometry combined with HPLC. Accell PAR1, PAR3, CXCR4 and non-targeting siRNA were purchased from GE Dharmacon.

**Plasmid construction**

pIRES-cMyc-hCXCR4var2-Rluc (CXCR4-Rluc) was kindly provided by Dr. Michel Bouvier. The FLAG-tagged Tango plasmid (F2RT-GANG or PAR1-TANGO, #66276) was from Addgene deposited by the laboratory of Dr. Bryan Roth (41). HA-tagged CXCR4 was as described(29,31). To construct the PAR1-EYFP fusion protein, cDNA encoding EYFP was amplified from pEYFP (Clontech Laboratories, Fremont, CA) and inserted at the restriction sites Age I and Xba I of PAR1-TANGO and fused to the C-terminus of PAR1. All plasmids were verified by DNA sequencing.

**Cells and cell lines**

hPPEC (ATCC, Manassas, VA, PCS-100-022) were cultured in vascular cell basal medium (ATCC, PCS-100-030) with endothelial cell growth kit-VEGF (ATCC, PCS-100-041). HEK293T cells (ATCC, CRL-11268) were cultured in DMEM. The HTLA cell line, a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene, was generously provided by the laboratory of Dr. Bryan Roth (41) and maintained in high glucose Dulbecco’s Modified’s Eagle Medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL hygromycin B, and 2 µg/mL puromycin. All cells were cultured in a humidified environment at 37°C, 5% CO2.

**Gene silencing by RNA interference**

hPPECs were transfected with non-targeting siRNA (siCTL), PAR1 siRNA, PAR3 siRNA or CXCR4 siRNA at a final concentration of 1 µM using Accell Delivery Media (GE Dharmacon) as previously described (27,29,30).

**In vitro endothelial cell permeability assays**
Permeability assays with hPPECs were performed as described previously (12). In brief, 96-well collagen-coated permeability assay plates were prehydrated for 15 min, 5x10^5 cells were seeded on each well and grown to a confluent monolayer for 48 hours. Fluorescein isothiocyanate (FITC)-dextran (20 μg/mL) was then added on top of the monolayer and the amount of FITC-dextran that permeated through the monolayer was quantified by measuring fluorescence in a Synergy 2 Multi-mode Microplate Reader (BioTek, Winooski, VT) at various time points over a 255 min time period.

Proximity ligation assays (PLAs)

PLAs were performed as described (12,27,29,31). The following primary antibodies were utilized for the detection of individual receptors and receptor-receptor interactions: rabbit anti-PAR-1 (Abcam, Cambridge, UK, ab63445), goat-anti-CXCR4 (Abcam, ab1670), and mouse anti-HA (ThermoFisher, 26183). All primary antibodies were used in a 1:750 volume/volume dilution. Comparisons and statistical analyses were performed only when PLA assays were performed on the same day in parallel experiments and fluorescence microscopy was performed with identical settings.

PRESTO-Tango β-arrestin-2 recruitment assay

The PRESTO-Tango assay was performed as previously described (12,29-31,41,42). HTLA cells (2.5 × 10^5/well) were seeded in a 6-well plate and transfected with 750 ng of each plasmid (PAR1-Tango plus pcDNA or PAR1-Tango plus HA-CXCR4) using Lipofectamine 3000 (ThermoFisher Scientific). The following day, transfected HTLA cells (75,000 cells/well) were plated onto Poly-L-Lysine pre-coated 96-well microplates and grown 24 hours. After cells were treated with thrombin overnight, culture media was removed and replaced with a 100 μL 1:5 mixture of Bright-Glo (Promega) and 1× HBSS, 20 mM HEPES solution. Plates were then incubated at room temperature for 20 min before measuring luminescence on a Biotek Synergy II plate reader. When HTLA cells were not transfected with a Tango plasmid, no change in luminescence was detectable upon agonist treatment.

Bioluminescence resonance energy transfer (BRET) assays

BRET assays were performed as described (30,59). In brief, HEK293T cells were seeded in 12-well plates and transfected with the plasmids indicated using the Lipofectamine 3000 transfection reagent (ThermoScientific). For BRET titration assays, CXCR4-RLuc at a fixed amount of 50 ng was transfected alone or with increasing amounts of EYFP or PAR1-EYFP. For BRET assays at a constant energy donor : acceptor ratio, increasing amounts of both CXCR4-RLuc and PAR1-EYFP were co-transfected at a ratio of 1:10. In all assays, empty vector pcDNA3 was added to maintain the total DNA amount for each transfection constant. After an overnight incubation, cells were seeded in poly-L-lysine coated 96-well white plates and incubated again overnight. Cells were then washed with PBS and fluorescence was measured in a Biotek Synergy HT4 plate reader (excitation 485 nm, emission 528 nm). For BRET measurements, coelenterazine H was added at a final concentration of 5 μM. After 10 min incubation at room temperature, luminescence was measured at 460 nm and 528 nm. The BRET signal was calculated as the ratio of the relative luminescence units (RLU) measured at 528 nm over RLU at 460 nm. The net BRET is calculated by subtracting the BRET signal detected when CXCR4-RLuc was transfected alone. For titration experiments, net BRET ratios are expressed as a function of fluorescence/total luminescence.

Immunoblotting

Immunoblotting with anti phospho-ERK1/2 ((Thr202/Tyr204), total ERK1/2 and anti-RhoA (all from Cell Signaling Technology) was performed as described (27,60,61). Densitometric quantifications of the band densities were performed with the Quantity One software (Bio-Rad, Hercules, CA).

Ras homolog gene family member A (RhoA)-GTP pull-down assays

RhoA-GTP pull-down assays were performed using the Active Rho Dectection Kit (Cell Signaling Technology) according to the manufacturer’s instructions. In brief, cells were serum starved for 5 hours, exposed to vehicle, the
TM2 peptide or CXCL12 for 30 min, and then treated with thrombin for 5 min. Cells were washed with ice-cold PBS and lysed in 25 mM Tris, pH 7.5, 250 mM NaCl, 0.05% Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, and 5 mM MgCl₂, supplemented with protease inhibitors. The cell lysate was incubated at 4°C for 1 h with GST-rhotekin RBD attached to glutathione-agarose beads. The beads were then washed three times with wash buffer (50 mM Tris, pH 7.5, 75 mM NaCl₂, 0.5% Triton X-100, and 5 mM MgCl₂) and boiled in SDS-PAGE sample buffer. Activated RhoA was estimated by comparing pull-down RhoA-GTP eluted from the agarose beads versus total RhoA in the cell lysates by immunoblotting.

**Flow cytometry**

Flow cytometry after labeling cells with Phycoerythrin-conjugated anti-FLAG (BioLegend, San Diego, CA, 637310), FITC-conjugated anti-HA (Sigma-Aldrich, H7411), or with rabbit anti-PAR1 (Abcam, Cambridge, UK, ab63445) and corresponding secondary Alexa 647- or Alexa 488 conjugated antibodies was used to quantify receptor expression levels in HTLA cells and in hPPECs, as previously described (30,31). At least 10,000 cells/sample were recorded and analyzed with the FlowJo software (Flowjo LLC, Ashland, OR 97520).

**Ca²⁺ assay**

Intracellular calcium was measured using the FLIPR Calcium 6 assay kit (Molecular Devices), as previously described (27,62,63).

**Data analyses**

Data are presented as mean ± standard error of n independent experiments performed on different days. Data were analyzed with unpaired Student’s t test, one- or two-way analyses of variance with Bonferroni’s multiple comparison post hoc test, as appropriate. Dose–response curves were generated using nonlinear regression analyses. All analyses were performed with the GraphPad-Prism 8, Version 8.4.0 (GraphPad Software, La Jolla, CA) software. A two-tailed p < 0.05 was considered significant.

**Data availability**

All data are contained within the manuscript.
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**Figure Legends**

**Figure 1:** PAR1 heteromerizes with CXCR4. **A.** HEK293T cells were transfected with a fixed amount of CXCR4-RLuc and increasing amounts of PAR1-EYFP (grey squares) or EYFP (open circles). 48 h after transfection, EYFP fluorescence and luminescence were read as described in Methods. Net BRET (528nm/460nm) was plotted against EYFP fluorescence/luminescence (EYFP/Lum). The Graph is representative of three independent experiments. **B.** HEK293T cells were transfected with increasing amounts of both CXCR4-RLuc and PAR1-EYFP at a fixed ratio (1:10) in quadruplicate. Raw BRET (528nm/460nm) was plotted against total DNA amounts transfected. N=3. **C.** hPPECs were transfected with PAR1 siRNA (red line) or non-targeting siRNA (blue line) and stained with anti-PAR1/Alexa-488 conjugated anti-rabbit. Black line: hPPECs labeled with Alexa-488 conjugated anti-rabbit (ctrl.). The graph is representative of n=3. **D.** hPPECs were incubated with non-targeting siRNA, PAR1 siRNA or PAR3 siRNA. PLA was performed for the detection of PAR1. Left: Representative PLA images for the detection of PAR1. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride signals. Scale bar: 25 µm. Right: Quantification of PLA signals. N = 3 with n = 10 images per condition and experiment. *: p<0.05 vs. cells transfected with non-targeting and PAR3 siRNA. **E.** Representative images for the detection of PAR1, CXCR4 and PAR1:CXCR4 heteromers by PLA in hPPECs. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride signals. Ctrl: hPPECs were incubated with anti-PAR1 alone and PLA performed for the detection of PAR1:CXCR4 heteromers. Scale bar: 25 µm. Images are representative of n = 5 experiments.

**Figure 2:** A peptide analogue of TM2 of CXCR4 interferes with PAR1:CXCR4 heteromerization. **A.** hPPECs were incubated with 50 µM of the TM2 or control TM peptides at 37 ºC for 30 min, followed by PLA for the detection of CXCR4, PAR1 and PAR1:CXCR4 heteromers. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride signals. Scale bar: 25 µm. Images are representative of three independent experiments. **B.** Quantification of PLA signals from three independent experiments, as in A. *: p<0.05 vs. vehicle and TM control. **C.** HEK293T cells were transfected with HA-CXCR4. 24 hours after transfection, cells were incubated with 50 µM of the TM2 peptide or vehicle at 37ºC for 30 min, followed by PLA for the detection of HA-CXCR4, PAR1 and PAR1:HA-CXCR4 heteromers. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride signals. Scale bar: 25 µm. Images are representative of three independent experiments. **D.** Quantification of PLA signals from three independent experiments, as in C. *: p<0.05 vs. vehicle.

**Figure 3:** CXCR4 reduces β-arrestin-2 recruitment to PAR1 and enhances thrombin-induced Ca²⁺ fluxes. **A.** HTLA cells were transfected with FLAG-PAR1-Tango plus pcDNA3 (blue line) or HA-CXCR4 (red line) and flow cytometry performed for the detection of FLAG-PAR1 with anti-FLAG (top) and of HA-CXCR4 with anti-HA (bottom). Grey area: unstained cells. **B.** PAR1 PRESTO-Tango β-arrestin recruitment assay upon stimulation with thrombin in cells transfected as in A. RLU: relative luminescence units. N=5. *: p<0.05 vs. cells transfected with PAR1-Tango/pcDNA3. **C.** PAR1 PRESTO-Tango β-arrestin recruitment assay upon stimulation with thrombin after pre-incubation with 20 µM of the TM2 peptide (grey symbols) or the control TM peptide (open symbols) at 37ºC for 30 min. Cells were transfected as in AB. N=3. RLU%: relative luminescence units in % of cells transfected with PAR1-Tango/pcDNA3,
pre-treated with the control TM peptide and stimulated with 1 µM thrombin (=100%). *: p<0.05 vs. cells transfected with PAR1-Tango/pcDNA3 pre-treated with the control TM peptide. D. HTLA cells transfected with FLAG-PAR1-Tango were incubated with vehicle (open circles, ctrl.) or 50 µM of the TM2 peptide (grey squares) at 37ºC for 30 min and calcium fluxes upon thrombin stimulation (arrows) measured. RFU: relative fluorescence units, expressed as % of baseline (=100%). N=3. E. HTLA cells were transfected with FLAG-PAR1-Tango plus pcDNA3 (open bars) or FLAG-PAR1-Tango plus CXCR4 (grey bars), as in A. Cells were pre-treated with 20 µM of the TM2 peptide or the control TM peptide at 37ºC for 30 min as indicated (+/-), and calcium fluxes upon thrombin stimulation (arrows) were measured as in D. Ca²⁺(dF/F0): maximal RFU minus baseline RFU (=dF) divided by baseline RFU (F0). N=4. *: p<0.05 vs. cells transfected with FLAG-PAR1-Tango plus pcDNA3 pre-treated with the control TM peptide.

Figure 4: CXCR4 modulates thrombin-induced ERK1/2 phosphorylation. A/C. HTLA cells were transfected with FLAG-PAR1-Tango plus pcDNA3 (A) or with FLAG-PAR1-Tango plus CXCR4 (C) as in Fig. 3. Forty-eight h after transfection, cells were serum starved for 6 h and then pre-treated with 20 µM of the TM2 peptide or the control TM peptide (TM ctrl.) at 37ºC for 30 min, followed by stimulation with 33 nM thrombin for 0-30 min. Cells were lysed and lysates used for the detection of phospho-ERK1/2 (top) and total ERK1/2 (bottom) by immunoblotting. Images are representative of 5 independent experiments. The migration position of molecular weight standards is indicated. B/D. Densitometric quantification of the band densities as in A/C. The phospho-ERK1/2/total ERK1/2 ratio is expressed as % of unstimulated cells (=100%, ctrl.). Open circles: cells pre-treated with the control TM peptide. Grey squares: cells pre-treated with the TM2 peptide. N=5. *: p<0.05 vs. cells pre-treated with the control TM peptide.

Figure 5: CXCR4 regulates thrombin-induced signaling in hPPECs. A. hPPECs were grown to 90% confluence and incubated with 20 µM of TM2 or control TM peptides at 37ºC for 30 min, followed by treatment with 33 nM of thrombin for 5 min. Cells were lysed and lysates used for the detection of phospho-ERK1/2 (top) and total ERK1/2 (bottom) by immunoblotting. Images are representative of 5 independent experiments. The migration position of molecular weight standards is indicated. B. Densitometric quantification of the band densities as in A. The phospho-ERK1/2/total ERK1/2 ratio is expressed as % of unstimulated cells (=100%, ctrl.). N=5. *: p<0.05 as indicated. C. CXCR4 gene silencing by RNA interference. hPPECs were incubated with non-targeting or CXCR4 siRNA. Surface expression of CXCR4 (left) and PAR1 (right) was measured by flow cytometry. Grey: unstained cells. Blue line: cells incubated with NT-siRNA. Red: cells incubated with CXCR4 siRNA. D. hPPECs after incubation with NT-siRNA or CXCR4 siRNA, as in C., were treated with vehicle (-) or 33 nM of thrombin (+) at 37ºC for 5 min. Detection of phospho-ERK1/2 (top) and total ERK1/2 (bottom) was performed by immunoblotting as in A. The image is representative of 3 independent experiments. The migration position of molecular weight standards is indicated. E. Densitometric quantification of the band densities as in D. The phospho-ERK1/2/total ERK1/2 ratio is expressed as % of unstimulated cells (=100%, ctrl.). N=3. *: p<0.05 for cells incubated with NT-siRNA vs. CXCR4 siRNA. F. RhoA-GTP pull-down assays. hPPEC were incubated with 50 µM of the TM2 peptide or 50 nM of CXCL12 at 37ºC for 30 min and then treated with 33 nM of thrombin for 5 min. Cells were lysed and lysates used for RhoA pull-down assays. Top: Immunoblot for the detection of RhoA in cell lysates. Bottom: Immunoblot for the detection of RhoA after RhoA-GTP pulldown. Images are representative of 3 independent experiments. The migration position of molecular weight standards is indicated. G. Densitometric quantification of the band densities as in F. The RhoA-GTP/total RhoA ratio is expressed as % of unstimulated cells (=100%, ctrl.). N=3. *: p<0.05 vs. ctrl.
% of hPPECs pre-treated with the control TM peptide and stimulated with vehicle (=100%, ctrl.). N=3. *: p<0.05 vs. ctrl. C. Quantification of PAR1 expression, as in A. PAR1 expression is expressed as % of hPPECs pre-treated with the control TM peptide and stimulated with vehicle (=100%, ctrl.). N=3. *: p<0.05 vs. ctrl.

**Figure 7: Effects of agonist stimulation on CXCR4, PAR1 and CXCR4:PAR1 heteromer expression in hPPECs.** A/B. hPPECs were pre-treated with 50 µM of the control TM (TM ctrl., A) or TM2 (B) peptides at 37ºC for 30 min, washed with PBS stimulated with vehicle, 50 nM of CXCL12 or 33 nM of thrombin for 30 min at 37ºC. Cells were then used for PLA for the detection of CXCR4, PAR1 and PAR1:CXCR4 heteromers. Images show merged PLA/4',6-diamidino-2-phenylindole dihydrochloride signals. Scale bar: 25 µm. Images are representative of three independent experiments. C-E. Quantification of PLA signals (C: CXCR4; D: PAR1; E: CXCR4:PAR1 heteromers) from three independent experiments, as in A/B. *: p<0.05 vs. cells pre-treated with the control TM peptide and stimulated with vehicle. #: p<0.05 vs. cells pre-treated with the TM2 peptide and stimulated with vehicle.

**Figure 8: A peptide analogue of TM2 of CXCR4 inhibits thrombin-induced impairment of hPPEC barrier function.** hPPECs cells were grown to a confluent monolayer on collagen coated permeable membranes. Cells were pretreated with TM2 (A) or control peptides (B) for 10 minutes, then exposed to thrombin (33 nM), followed by the addition of FITC-labelled dextran. Endothelial permeability was assessed by monitoring the amount of FITC-labelled dextran that permeated through the cell monolayer by measuring fluorescence in the solution underneath the membrane insert at different time points. RFU: Relative fluorescence units. N = 3 in quadruplicate. *: p<0.05 vs. thrombin alone. #: p<0.05 vs. cells pre-treated with 50 µM of the TM2 peptide followed by thrombin exposure.
Figure 1

A. net BRET (528/460 nm) vs. EYFP/Lum

B. BRET (528/460 nm) vs. DNA (µg)

C. Distribution of cells with NT siRNA, PAR1 siRNA, and ctrl.

D. PLA signals (% NT) for NT siRNA, PAR1 siRNA, and PAR3 siRNA

E. Confocal images of PAR1 and CXCR4: PAR1:CXCR4 and ctrl.
Figure 2
Figure 4

**A**

PAR1 + pcDNA3

|       | TM ctrl. | TM2 |
|-------|----------|-----|
| 0     | 5        | 10  |
| 20    | 30       |     |

- **pERK1/2**
- **ERK1/2**

**B**

![Graph showing pERK1/2/ERK1/2 (% ctrl.) over time for PAR1 + pcDNA3 - TM ctrl. and PAR1 + pcDNA3 - TM2.](graph1.png)

**C**

PAR1 + CXCR4

|       | TM ctrl. | TM2 |
|-------|----------|-----|
| 0     | 5        | 10  |
| 20    | 30       |     |

- **pERK1/2**
- **ERK1/2**

**D**

![Graph showing pERK1/2/ERK1/2 (% ctrl.) over time for PAR1 + CXCR4 - TM ctrl. and PAR1 + CXCR4 - TM2.](graph2.png)

* indicates a significant difference.
Figure 5
Figure 6

A) Flow cytometry analysis of PAR1 and CXCR4 expression in TM ctrl. and TM2 cells treated with CXCL12 or thrombin.

B) Bar graph showing CXCR4 expression (% ctrl.) under different conditions:
- TM ctrl.: +
- TM2: - +
- CXCL12: - + + - - +
- Thrombin: - - - + +

C) Bar graph showing PAR1 expression (% ctrl.) under different conditions:
- TM ctrl.: + - + - + -
- TM2: - + + - - +
- CXCL12: - + + + -
- Thrombin: - - - + +

* Significant difference compared to control.
Figure 7

(A) TM ctrl.

CXCR4

PAR1

CXCR4:PAR1

(B) TM2

CXCR4

PAR1

CXCR4:PAR1

(C) CXCR4 - PLA signals/cell

(D) PAR1 - PLA signals/cell

(E) CXCR4:PAR1 - PLA signals/cell

| Condition   | TM ctrl. | TM2 | CXCL12 | Thrombin |
|-------------|----------|-----|--------|----------|
| CXCR4       | +        | -   | -      | -        |
| PAR1        | -        | +   | -      | -        |
| CXCR4:PAR1  |          |     | +      | +        |

* Significant difference

# Significant difference
Figure 8

A. no cells (100% permeability)
- no treatment
- TM2 (50 µM)
- Thrombin (33 nM)
- Thrombin + TM2 (5 µM)
- Thrombin + TM2 (50 µM)

B. no cells (100% permeability)
- no treatment
- TM ctrl. (50 µM)
- Thrombin (33 nM)
- Thrombin (33 nM) + TM ctrl. (50 µM)
Regulation of the thrombin/protease-activated receptor 1 axis by chemokine (C-X-C motif) receptor 4
Xianlong Gao, You-Hong Cheng, Garrett A Enten, Anthony J DeSantis, Vadim Gaponenko and Matthias Majetschak

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