

Supporting Information

Caccuri et al. 10.1073/pnas.1206605109

SI Materials and Methods

Recombinant HIV-1 p17 Protein and mAb to p17. Purified endotoxin (LPS)-free recombinant HIV-1 matrix protein p17 (in its monomeric form) and GST were produced as previously described (1). The absence of endotoxin contamination (<0.25 endotoxin units/mL) in protein preparations was assessed by Limulus amoebocyte assay (Associates of Cape Cod). The p17 neutralizing mAb MBS-3 was produced in our laboratory (2).

Cell Cultures. Human umbilical vein endothelial cells (HUVECs) were isolated and characterized as previously described (3) and cultured in endothelial growth medium (EGM) (Lonza) containing 10% (vol/vol) FBS. All experiments were carried out with cells at passages 2–6. When reported, HUVECs were pretreated for 2 h at 37 °C with 15 mU/mL of HEP-II (Sigma-Aldrich) or cultured in medium containing 7.3 nM of 13.6 kDa heparin (Laboratori Derivati Organici).

Surface Plasmon Resonance (SPR) Binding Assay. SPR measurements were performed on a BIAcore X instrument (GE Healthcare) as previously described (4). Anti-GST antibody was immobilized on a CM5 surface (GE Healthcare) using standard amine-coupling chemistry. Then, recombinant human CXCR2 with a C-terminal GST tag [10 µg/mL in running buffer composed of Hepes 50 mM pH 7.0, 0.01% CHS, 0.1% CHAPS, and 0.33 mM synthetic phospholipid blend (dioleoyl) DOPC:DOPS (7:3 wt/wt, Avanti polar lipids)] was injected over the anti-GST surface at a flow rate of 5 µL/min, allowing the immobilization of about 1,600 Resonance Unit (equal to about 0.025 pM). A sensor chip coated with anti-GST antibody alone was used as a negative control and for blank subtraction. The immobilization of the receptor to the sensor chip was confirmed by injecting increasing concentrations of mAb to CXCR2 in running buffer containing 0.1 mg/mL BSA at a flow rate of 30 µL/min and evaluating its binding to CXCR2. IL-8, p17, and SDF-1α were injected over the CXCR2 or control surfaces as described for the CXCR2 mAb. Kinetic parameters were obtained from the sensorgram overlays by using the non-linear fitting (single site model) software package BIAevaluation 3.2. In the experiments shown, the fitting values of chi square were lower than 6.6.

In Vitro Tube Formation Assay. Prechilled 48-well culture plates were coated with 150 µL per well of basement membrane extract (BME) (10 mg/mL) (Cultrex; Trevigen) and incubated for 30 min at 37 °C. HUVECs were nutrient starved for 24 h in endothelial basal medium (EBM) containing 0.5% FBS (sEBM) for 24 h before they were harvested and resuspended in endothelial growth medium (EGM). Cells were seeded in the BME-coated plate (5×10^4 per well) and treated or not with 10 ng/mL of p17, IL-8, or GST. When reported, p17 was preincubated for 30 min at 37 °C with 1 µg/mL of unrelated control mAb (Ctrl Ab) or mAb MBS-3. Starved HUVECs were cultured, when indicated, with an isotype-matched mAb, a neutralizing mAbs to CXCR1 (MAB330), and/or a neutralizing mAb to CXCR2 (MAB331) (R&D). HUVECs were also pretreated, when indicated, with the inhibitor of MAPK kinase PD98059 (10 µM) (Calbiochem), the inhibitors of phosphatidylinositol 3-kinase (PI3-K) wortmannin (100 nM), and LY294002 (10 µM) (Enzo) or the Akt inhibitor VIII (1 µM) (Sigma-Aldrich). Wells were then analyzed over time (from 2 to 24 h) for the formation of tubule structure by examination with a Leica DM IRB microscope. The center of each well was

digitally photographed with a Hitachi KP-D50 camera and capillary-like structures were quantified by analyzing the number of tubes per well formed by HUVECs.

Western Blot Analysis. HUVECs (1×10^6) were starved for 24 h, stimulated with p17 10 ng/mL or GST 10 ng/mL at different time points (5, 15, 30, and 60 min) and then lysed in 200 µL of lysis buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM EDTA, 0.6% Nonidet P-40], containing a mixture of protease inhibitors (Complete Mini; Roche) and phosphatase inhibitors [1 mM sodium orthovanadate, 20 µM phenylarsine oxide (PAO) and 30 mM sodium fluoride; Sigma]. Equal amounts of total proteins were resolved on a 12% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The blots were incubated overnight at 4 °C with mAb to pAkt (Cell Signaling Technology), mAb to pERK1/2, or rabbit polyclonal Ab to ERK1/2 (Santa Cruz Biotechnology). The antigen-antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-conjugated goat antirabbit IgG or goat antimouse IgG (Thermo Scientific) and revealed using the ECL System (Santa Cruz Biotechnology).

Aortic Ring Assay. The rat aortic ring assay was performed as previously described (5). Briefly, the dorsal aorta was excised from 6-wk-old Sprague-Dawley rats. Rings, 1-mm thick, were embedded in collagen gel and then incubated with serum-free EBM containing p17 (20 ng/mL), GST (20 ng/mL), or VEGF-A (20 ng/mL). The plates were incubated for 10 d and angiogenesis was quantified by counting the number of microvessels originating from aortic rings.

Chick Chorioallantoic (CAM) Assay. Fertilized White Leghorn chicken eggs (30 per group) were incubated at 37 °C at constant humidity. On day 3, a square window was opened in the shell, and 2–3 mL of albumin was removed to allow detachment of the developing CAM. The window was sealed with a glass, and the eggs were returned to the incubator. On day 8, eggs were treated with 1 mm³ sterilized gelatin sponges (Gelfoam; Upjohn) placed on top of the growing CAM, as previously described (6) and loaded with 1 µL of PBS (negative control), 1 µL of PBS containing 200 ng of GST, 1 µL of PBS containing 200 ng of VEGF-A (positive control), or 1 µL of PBS containing 200 ng of p17. CAMs were examined daily until day 12 and photographed in ovo with a stereomicroscope equipped with a camera and image analyzer system (Olympus). At day 12, the angiogenic response was evaluated by the image analyzer system and counted as the number of vessels converging toward the sponge.

Immunohistochemistry. Liver tissues were obtained from a control patient and from a HIV-1-infected patient undergoing therapeutic surgery, according to the principles listed in the Helsinki Declaration. The HIV-1-infected patient was aviremic at the time of surgery and tested negative for proviral DNA in the liver biopsy. Four-micrometer formalin-fixed and paraffin-embedded serial sections were transferred to glass slides coated with polylysine and rehydrated by immersion in 100% xylene, followed by graded ethanol (100, 95, 90, 80, and 70% vol/vol). To enhance antigenicity and allow epitope unmasking, the sections were heat treated using the Bond Max automated autostainer (Menarini). Endogenous peroxidase was inhibited by incubation of tissue sections with 3% (vol/vol) hydrogen peroxide for 15 min at room

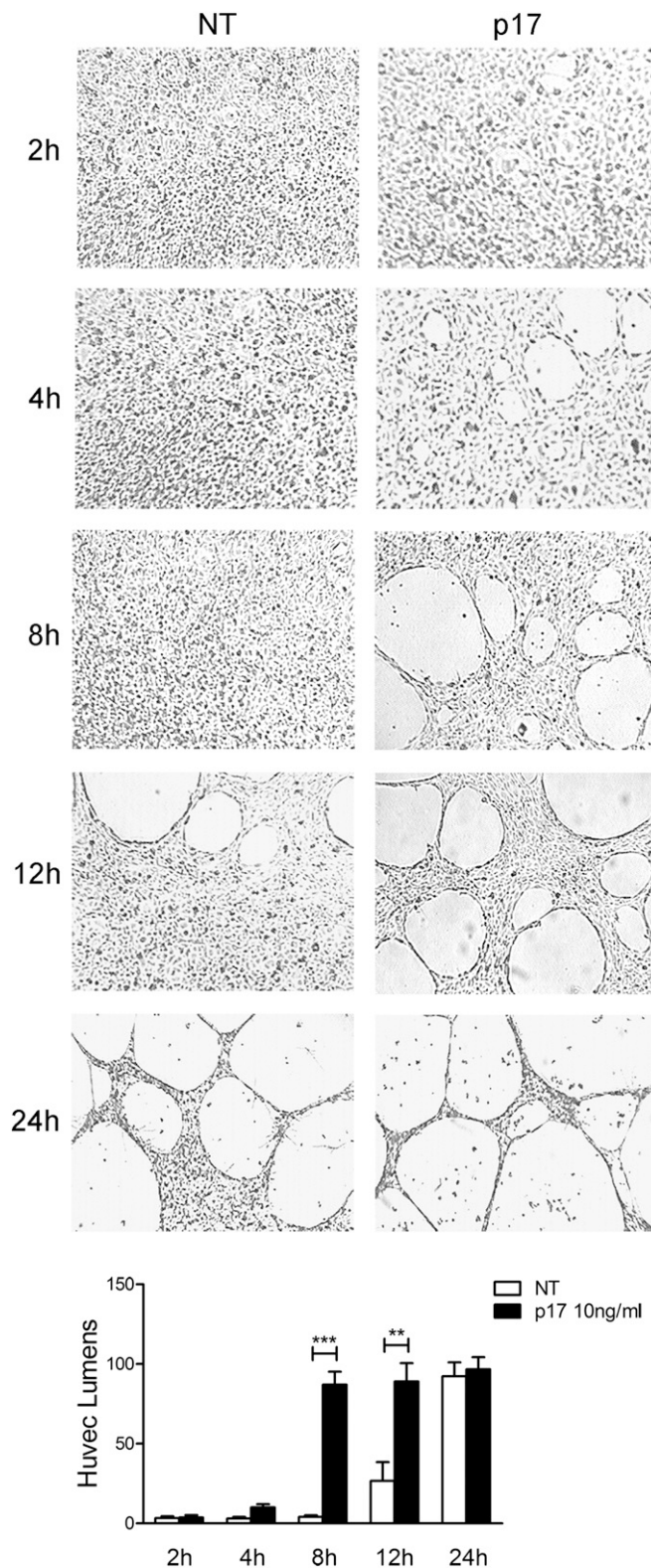


Fig. S1. Effects of p17 stimulation on organization of HUVECs in mEGM. Pictures were taken after 2, 4, 8, 12, and 24 h incubation at 37 °C (magnification 10×). P17 (10 ng/mL) promotes the organization of HUVECs into capillary-like networks. The effect starts at 4 h, is evident at 8 h of culture, and remains statistically significant also after 12 h of stimulation. On the contrary, HUVECs not stimulated with p17, but cultured up to 8 h, form a cellular monolayer with absence of capillary-like structure. Pictures are representative of three independent experiments with similar results. Values reported for HUVEC tubes are the mean ± SD of three independent experiments. Statistical analysis was performed by paired two-tail Student *t* test; ***P* < 0.01, ****P* < 0.001.

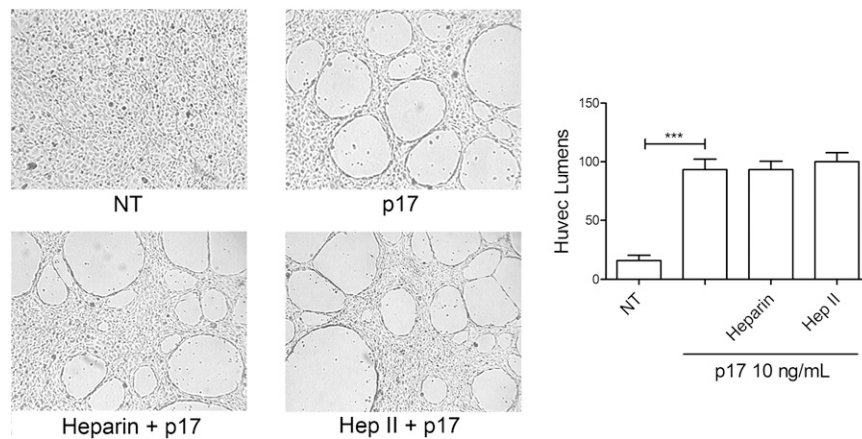


Fig. S2. P17-induced tube formation is not influenced by binding to heparan sulfate proteoglycan (HSPG). Angiogenic response of HUVECs to the indicated treatments. Pictures were taken after 8-h incubation at 37 °C (magnification 10x). When indicated HUVECs were pretreated for 2 h at 37 °C with heparinase II (Hep-II) (15 mU/mL) or cultured in medium containing 7.3 nM of heparin. Cells were stimulated with PBS (not treated cells, NT) or p17 (10 ng/mL). Pictures are representative of three independent experiments with similar results. Values reported for HUVEC tubes are the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA and the Bonferroni posttest was used to compare data; ****P* < 0.001.

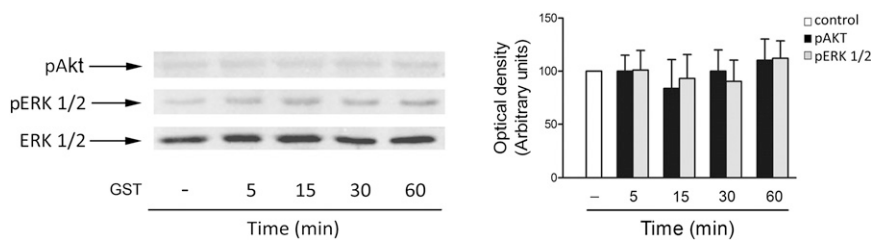


Fig. S3. Effects of GST stimulation on Akt and ERK1/2 activity in ECs. HUVECs were treated or not for 5, 15, 30, and 60 min with GST (10 ng/mL). EC lysates were evaluated for expression of ERK1/2, pERK1/2, and pAkt by Western blot analysis using a rabbit polyclonal Ab to ERK1/2, or mAb to pERK1/2, or pAkt as specific reagents. Phosphorylation of ERK1/2 and Akt was verified by densitometric analysis and plotting of the pERK1/2/ERK1/2 and pAkt/ERK1/2. (Left) Blots from one representative experiment of three with similar results are shown. (Right) Values reported for pERK1/2 and pAkt are the mean ± SD of three independent experiments.

Table S1. Binding parameters of the interaction of mAb anti-CXCR2, p17, IL-8, and SDF-1α with CXCR2

	CXCR2		
	Association rate (Kon, 1/Ms)	Dissociation rate (Koff, 1/s)	Dissociation constant (K_d , nM)
Anti-CXCR2 antibody	9.7×10^4	0.24×10^{-3}	2.5
p17	8.2×10^4	10.4×10^{-3}	130.0
IL-8	2.8×10^4	1.9×10^{-3}	70.0
SDF-1α	ND	ND	ND

Association rate (Kon) and dissociation rate (Koff) are reported and the dissociation constant (K_d) was derived from the Koff/Kon ratio. Results are representative of two independent experiments with similar results. K_d values were also calculated independently from binding kinetics, by performing Scatchard plot analysis of the equilibrium binding data. ND, not determinable.