BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

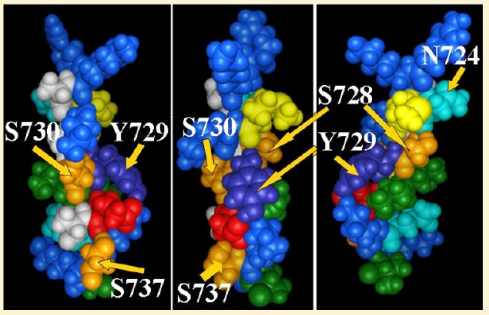
pubs.acs.org/biochemistry

Distinct Sites within the Vascular Cell Adhesion Molecule-1 (VCAM-1) Cytoplasmic Domain Regulate VCAM-1 Activation of Calcium Fluxes versus Rac1 during Leukocyte Transendothelial Migration

Michelle E. Marchese, Sergejs Berdnikovs, and Joan M. Cook-Mills*

Division of Allergy and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, United States

ABSTRACT: Vascular adhesion molecules regulate the migration of leukocytes from the blood into tissue during inflammation. Binding of leukocytes to vascular cell adhesion molecule-1 (VCAM-1) activates signals in endothelial cells, including Rac1 and calcium fluxes. These VCAM-1 signals are required for leukocyte transendothelial migration on VCAM-1. However, it has not been reported whether the cytoplasmic domain of VCAM-1 is necessary for these signals. Interestingly, the 19-amino acid sequence of the VCAM-1 cytoplasmic domain is 100% conserved among many mammalian species, suggesting an important functional role for the domain. To examine the function of the VCAM-1 cytoplasmic domain, we deleted the VCAM-1 cytoplasmic domain or mutated the cytoplasmic domain at amino acid N724, S728, Y729, S730, or S737. The cytoplasmic domain and S728, Y729, S730, or S737 were necessary for leukocyte transendothelial migration. S728 and Y729, but not S730 or S737, were necessary for VCAM-1 activation of calcium fluxes. In contrast, S730 and S737, but not S728 or Y729, were necessary for VCAM-1 activation of Rac1. These functional data are consistent with our computational model of the structure of the VCAM-1 cytoplasmic domain as an α-helix with S728 and Y729, and S730 and S737, on opposite sides of the α-helix. Together, these data indicate that S728 and Y729, and S730 and S737, are distinct functional sites that coordinate VCAM-1 activation of calcium fluxes and Rac1 during leukocyte transendothelial migration.



During inflammation, expression of vascular cell adhesion molecule-1 (VCAM-1) is induced on endothelial cells. VCAM-1 regulates the recruitment of leukocytes into inflamed tissues. Its ligands include the high-affinity conformation of integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ expressed on lymphocytes, eosinophils, mast cells, and monocytes.[1−7] Chemokine receptors induce activation of these integrins to the high-affinity conformation on T cells, B cells, mast cells, eosinophils, or monocytes for migration on VCAM-1. Thus, the cell types that migrate on VCAM-1 during an immune challenge depend on the cell type specific chemokines in the microenvironment. VCAM-1 regulates eosinophil migration in asthmatic inflammation, T cell trafficking through the blood−brain barrier during multiple sclerosis, and the development of atherosclerotic plaques in cardiovascular disease.[8−10] Additionally, VCAM-1$^{-/-}$ mice are embryonic lethal, because of impaired placental and heart development.[11] Binding to VCAM-1 activates a signaling cascade in endothelial cells that results in endothelial cell retraction, allowing the passage of leukocytes from the blood to the tissue.[12−19]

VCAM-1 signaling is necessary for VCAM-1-dependent leukocyte migration. Upon antibody cross-linking of VCAM-1, endothelial cell intracellular calcium is released, calcium channels are activated, and Rac1 is activated. Together, calcium fluxes and Rac1 activate endothelial NOX2.[15,20,21] NOX2 generates low levels of superoxide that dismutates to H_2O_2. This H_2O_2 activates matrix metalloproteinases in the endothelial extracellular matrix. The H_2O_2 also diffuses through the endothelial membrane to oxidize and activate protein kinase Cα (PKCα).[14,17] PKCα activation leads to the phosphorylation of protein tyrosine phosphatase 1B (PTP1B), which then induces signals for ERK1/2 phosphorylation.[18,22] Blocking any of these signals disrupts VCAM-1-dependent leukocyte transendothelial migration.

While several intermediates in the VCAM-1 signaling cascade have been reported, it has not been determined whether the VCAM-1 cytoplasmic domain is necessary for the initiation of these signals. The 19-amino acid VCAM-1 cytoplasmic domain is highly conserved among species. This level of interspecies conservation suggests an important functional role for the VCAM-1 cytoplasmic domain. Therefore, we hypothesized that the VCAM-1 cytoplasmic domain initiates the VCAM-1 signaling cascade. In this report, we demonstrate that the cytoplasmic domain is necessary for rapid VCAM-1 signals and VCAM-1-dependent leukocyte transendothelial migration. Furthermore, by creating single-amino acid point mutations, we identified two amino acids in the VCAM-1 cytoplasmic domain that are necessary for VCAM-1 activation of Rac1 and two distinct amino acids that are necessary for VCAM-1 activation of calcium, allowing leukocyte transendothelial migration.

Received: July 11, 2012
Revised: September 7, 2012
Published: September 12, 2012

   dx.doi.org/10.1021/bi300925r | *Biochemistry* 2012, 51, 8235−8246

MATERIALS AND METHODS

Cells. Endothelial cell line mHEVa was derived from the axial lymph nodes of male BALB/c mice and cultured in RPMI medium supplemented with 20% FCS, 2 mM glutamine, 1 mM HEPES, 10 mM sodium bicarbonate, 100 units/mL penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamicin as previously described.[23] The mHEVa cell line was spontaneously immortalized but not transformed. The mHEVa cell line constitutively expresses VCAM-1 but does not express adhesion molecule ICAM-1, MAdCAM, or PECAM-1 as determined by flow cytometry and microarray analysis.[23] Human umbilical vein endothelial cells were from Lonza (catalog no. CC-2517). Spleen leukocytes were obtained from BALB/c male mice. Animal procedures were reviewed and approved by the Animal Care and Use Committee of Northwestern University.

Reagents. Rat anti-mouse VCAM-1 (clone MVCAM.A) was from eBioscience (catalog no. 550547). FITC-conjugated mouse anti-human ICAM-1 (clone 84H10, catalog no. MCA532F) and mouse anti-human ICAM-1 (clone 84H10, catalog no. MCA532) were from AbD Serotec. Goat anti-mouse IgG1 (catalog no. 1070-01), goat anti-rat IgG (catalog no. 3050−01), biotin-conjugated goat anti-rat IgG (catalog no. 3030-08), biotin-conjugated goat anti-mouse IgG (catalog no. 1034), rat IgG isotype control (catalog no. 0108-01), and mouse IgG isotype control (catalog no. 0107-01) were from Southern Biotech. FITC-conjugated goat anti-rat IgG (catalog no. 554016) and rat anti-mouse CD16/CD32 (clone 2.4G2, catalog no. 553142) were from BD Pharmigen. The pcDNA3.1 V5-His-TOPO TA expression kit was from Invitrogen (catalog no. K4800). Transfection was performed using TransIT-LT1 transfection reagent (Mirus, catalog no. MIR2300). The QuikChange II Site-Directed Mutagenesis Kit (catalog no. 200524) was from Agilent Technologies. The Halt Protease/Phosphatase inhibitor cocktail was from Thermo Scientific (catalog no. 78441).

Antibody-Coated Beads. For anti-mouse VCAM-1-coated beads, streptavidin-coated 9.9 μm diameter beads (80 μL) (Bangs Laboratories) were labeled with 24 μg of biotin-conjugated goat anti-rat IgG in 375 μL of PBS with gentle rocking for 1 h at 4 °C and then washed three times.[24] These beads were incubated with 16 μg of rat anti-mouse VCAM-1 or a rat isotype IgG control antibody in 375 μL of PBS with gentle rocking for 1 h at 4 °C and then washed. For anti-human ICAM-1-coated beads, the streptavidin-conjugated beads were coated with 24 μg of biotin-conjugated goat anti-mouse IgG and then 16 μg of mouse anti-human ICAM-1 or a mouse isotype IgG control antibody.

Plasmid with the V/I Chimeric Receptor. The V/I chimera was created by ligating in frame the cDNA encoding the first two immunoglobulin-like domains of human ICAM-1 to the cDNA encoding human VCAM-1 immunoglobulin-like domains 3−7, the transmembrane region, and the cytoplasmic domain of VCAM-1. For this construct, the mRNA for VCAM-1 and ICAM-1 was isolated from TNF-stimulated primary cultures of human umbilical vein endothelial cells and amplified by polymerase chain reaction. The primers for full-length, 7-Ig-like domain human VCAM-1 were as follows: forward, AATTAGGTACCACACACAGGTGGGACACAAA; reverse, ATATACTCGAGTCTCCAGTTGAACATATCAAGCA. The primers for immunoglobulin-like domains 1 and 2 of human ICAM-1 were as follows: forward, AATTAGGATCCCAGTCGACGCTGAGCTCCTCTGCTA; reverse, ATATAGATATCAAAGGTCTGGAGCTGGTAGGG.

Full-length VCAM-1 was digested with restriction enzymes to remove immunoglobulin-like domains 1 and 2. The remaining VCAM-1 and ICAM-1 sequences were ligated into the pcDNA 3.1 V5-His-TOPO (Invitrogen, catalog no. K4800) backbone plasmid to create the wild type VCAM-1/ICAM-1 (V/I WT) chimera with a CMV promoter and Geneticin resistance gene. To ensure accuracy, the plasmid was completely sequenced at the Northwestern University Genomics Core Facility.

Plasmid with V/I Receptor Mutations. The following mutants were created using the V/I chimera plasmid described above and the Agilent Technologies QuikChange II Site-Directed Mutagenesis kit with the following primers: mutant S728A (GAAAAGCCAACATGAAGGGGGCATATAGTCTTGTAG) and reverse (CTACAAGACTATATGCCCCCTTCATGTTGGCTTTTC), mutant Y729F (GCCAACATGAAGGGGGTCAGCTAGTCTTGTAGAAGCAC) and reverse (GTGCTTCTACAAGACTAGCTGACCCCTTCATGTTGGC), mutant S730A (GCCAACATGAAGGGGTCATATGCTCTTGTAGAAGCACAGAAATC) and reverse (GATTTCTGTGCTTCTACAAGAGCATATGACCCCTTCATGTTGGC), mutant S737A (CTTGTAGAAGCACAGAAAGCAAAAGTGTAGCTAATGCTTG) and reverse (CAAGCATTAGCTACACTTTTGCTTTCTGTGCTTCTACAAG), whole tail deletion (GCAAGAAAAGCCTAGATGAAGGGGTCATATAGTCTTGTAGAAGC) and reverse (GCTTCTACAAGACTATATGACCCCTTCATCTAGGCTTTTCTTGC), and mutant N724A (CTTTGCAAGAAAAGCCGCCATGAAGGGGTCATATAG) and reverse (CTATATGACCCCTTCATGGCGGCTTTTCTTGCAAAG). To ensure accuracy, each resulting plasmid was fully sequenced at the Northwestern University Genomics Core Facility.

Transfection and Cloning. The mHEVa cells were grown to 70% confluence in six-well plates and treated with the TransIT-LT1 transfection reagent and linearized pcDNA 3.1 V5-His-TOPO plasmids according to the manufacturer's protocol. Twenty-four hours after transfection, Geneticin selection medium was added at a concentration of 0.35 mg/mL (as previously determined by a cell death dose curve). Transfected cells were subcloned into 96-well plates and grown to 90% confluence before being labeled with FITC-conjugated anti-human ICAM-1. V/I construct expression was determined by immunolabeling with anti-ICAM-1 and flow cytometry or fluorescent microscopy. Positively expressing cells were selected and re-subcloned at least four times to ensure clonality. Two individually generated clones were selected for each construct. To ensure accuracy, the V/I chimeras in the clones were completely sequenced by the Northwestern University Genomics Core Facility.

Rac1 Activation. We followed the protocol from the Millipore Rac1 Activation Assay Kit (catalog no. 17-283). mHEVa cells were grown to confluence in 35 mm plates in mHEV culture medium.[23] To cross-link VCAM-1 or the V/I construct, primary and secondary antibodies were premixed for 5 min at room temperature (54 μg of anti-VCAM-1 with 30 μg of rat anti-mouse IgG1, or 27 μg of anti-ICAM-1 with 15 μg of mouse anti-human IgG1). mHEVa monolayers were stimulated by antibody cross-linking of VCAM-1 or the V/I construct for 30, 60, or 120 s. The medium was then immediately removed from the cells; 500 μL of lysis buffer was added, and the cells were snap-frozen in an ethanol/dry ice bath. The frozen culture dishes were placed on ice to thaw, and the cells were scraped from the dishes and placed in ice-cold Eppendorf tubes. The

samples were precleared with protein agarose G beads (Millipore, catalog no. 16-266); 35 μL of each sample was collected in a separate Eppendorf tube to determine the total amount of Rac1 per sample by Western blot. The remainder of each sample was added to 10 μL of Pak-1 PBD beads. The samples were rocked for 2 h at 4 °C, and then the beads were washed three times with a magnesium lysis buffer (Millipore, catalog no. 20-168). Laemmli buffer (Bio-Rad, catalog no. 161-0737) with 5% β-mercaptoethanol was added to the beads. The samples were boiled for 5 min and loaded onto a sodium dodecyl sulfate−polyacrylamide gel. Cell lysates or immunoprecipitates were loaded into Bio-RAD 10% Mini-PROTEAN TGX precast gels and were run at 130 V using the Bio-Rad Mini-PROTEAN system. Gels were transferred to nitrocellulose membranes using semidry transfer with the Bio-Rad SemiDry apparatus. Membranes were blocked in 5% milk in 1% TBS-Tween, and a Rac1 monoclonal antibody was added to the milk/TBS-Tween mixture for rocking overnight at 4 °C. After three washes in TBS-Tween, a secondary goat anti-mouse IR 800 (Rockland, catalog no. 610-132-121) antibody was added at a 1:5000 dilution and the membrane was incubated in 5% milk in 1% TBS-Tween for 1 h at room temperature. The membrane was washed three times in 1% TBS-Tween. The membrane was analyzed using the Odyssey Infrared Imaging System (LiCor).

Calcium Fluxes. Cells were grown to confluence in 96-well plates and loaded using the Fluo-4 NW Calcium Assay Kit (Invitrogen, catalog no. F36206) as detailed in the manufacturer's protocol. Briefly, the Fluo-4 was resuspended in HBSS with 20 mM probenecid to a concentration of 10× (HBSS and probenecid were provided in the kit). The Fluo-4/probenecid solution was diluted to 1× in phenol red free HEV medium.[25] The medium from the cells (spent medium) was removed from each well, and 100 μL of the 1× fluo-4/probenecid solution was added to each well. The plates were incubated for 30 min at 37 °C and for an additional 10 min at room temperature before the fluo-4 solution was replaced with spent medium. Spent medium was used rather than fresh culture medium because in fresh culture medium, serum growth factors stimulate growth factor receptor-mediated calcium fluxes. For cells stimulated with mouse anti-ICAM-1 or mouse isotype control antibody, the wells were pretreated with 4 μg of Fc block per 100 μL of medium (100 μL of medium per well). The cells were stimulated with 8×10^6 beads (Bangs Laboratories, catalog no. CP01N) coated with rat anti-VCAM-1 antibodies, mouse anti-ICAM-1 antibodies, mouse IgG isotype control antibodies, or rat IgG isotype control antibodies.[26] The relative Fluo-4 fluorescence was examined using a 7620 Microplate Fluorimeter (Cambridge Technology, Inc.). For each experiment, treatments were conducted in quadruplicate wells and calculated as an average minus background. Experiments were conducted three times.

Lymphocyte Migration Assay under Laminar Flow. The migration assay was performed as previously described.[12] Briefly, endothelial cells were grown to 100% confluence on Nunc Slide-flasks (Thermo Scientific, catalog no. 177410). Spleen cells were used as a source of cells contiguous with the bloodstream that could then migrate across endothelial cells. The migration of spleen cells across the mHEV cell lines is stimulated by mHEV cell constitutive production of chemokine MCP-1[27] and is dependent on adhesion to VCAM-1.[26] Splenocytes were isolated from BALB/c male mice. Red blood cells were lysed by hypotonic lysis. The spleen leukocytes were >90% lymphocytes as previously reported.[12] The slides were not treated or pretreated with blocking anti-VCAM-1 (81 μg/slide) or anti-ICAM-1 (50 μg/slide) antibody in 1 mL of culture medium as indicated for 15 min prior to running the assay. The amount of function blocking antibody used per slide was determined by a dose curve with the wild type V/I chimera cell lines (data not shown). Then, the flask was removed from the slide, and the slide was placed in a parallel plate flow chamber with laminar flow. In vivo, in the absence of inflammation, the rapid fluid dynamics of the blood result in blood cells located midstream of the vascular flow.[28] However, during inflammation, there is a change in fluid dynamics.[28−30] With inflammation, the vascular permeability increases, yielding a flow of fluid from the blood into the tissues that likely contributes to the contact of blood cells with the endothelium ("margination").[28,30] There is also cell contact as the blood cells leave the capillaries and enter the postcapillary venules.[29] Therefore, spleen cells (3×10^6) were added to the flow chamber at a level of 2 dyn/cm^2. Then, to initiate the contact of spleen cells with the endothelial cells in vitro, the spleen cells were allowed to settle for 5 min in the chamber as monitored by microscopy and then 2 dyn/cm^2 was applied for the 15 min to mimic postcapillary blood flow.[12] We have observed by microscopy that during the assay under laminar flow, the spleen cells in contact with the endothelial cells either roll, roll and detach, or roll, firmly attach, and migrate. After cell contact, the focus of the studies is on mechanisms of transendothelial migration under conditions of laminar flow. For this assay, the coculture was exposed to laminar flow at 2 dyn/cm^2 at 37 °C for 15 min to examine cell migration. Then, the cells were washed with PBS supplemented with 0.2 mM $CaCl_2$ and 0.1 mM $MgCl_2$ because cations are required for cell adhesion. These cells were fixed with 3% paraformaldehyde for 1 h. Then the slides were coverslipped with gelvatol. To quantify migrated spleen cells, phase contrast microscopy was used to count migrated cells that are phase dark.[31] In each experiment, five high-powered fields were counted for each slide. The data are presented as the average ± standard error from three slides per treatment. In this assay, the endothelial cells constitutively produce chemokine MCP-1 to induce the transendothelial migration of leukocytes on VCAM-1.[12] It has been reported that the transendothelial migration of an individual leukocyte, after it has rolled to a site of migration, occurs in 2 min.[25] However, transendothelial migration of leukocytes is asynchronous. In the laminar flow assay, spleen cell migration is detected by 15 min. The number of spleen cells that were associated but not migrated (phase light cells) at 15 min is low because in 15 min, the majority of nonmigrating cells roll off the monolayer of endothelial cells as determined by microscopy (data not shown). Therefore, the number of spleen cells initially associated with the endothelial cells was determined in an adhesion assay.

Lymphocyte Adhesion Assay. The adhesion assay was performed as previously described.[12] Briefly, mHEVa cells were grown to confluence in flat-bottom, tissue culture-treated 96-well plates. Splenocytes were isolated as described in Lymphocyte Migration Assay under Laminar Flow and then labeled with calcein acetoxymethyl ester (calcein-AM) (1 μM) at 37 °C for 15 min and washed three times in PBS. These leukocytes (1×10^6 per well) were added to the mHEVa monolayers. For cell contact, the plate was incubated at 37 °C for 5 min. To remove nonbound leukocytes, the plates were washed three times with PBS with 200 μM $MgCl_2$ and 150 μM

dx.doi.org/10.1021/bi300925r | Biochemistry 2012, 51, 8235−8246

$CaCl_2$. The fluorescence was read using a 7620 Microplate Fluorometer (Cambridge Technology, Lexington, MA). A standard curve of fluorescence versus the number of leukocytes was used to calculate the number of leukocytes bound to the endothelial cells. To assess the binding of leukocytes to VCAM-1, the V/I construct was blocked with a blocking anti-ICAM-1 antibody (3 μg of antibody/100 μL of medium; MCA532, AbD Serotec). Likewise, to assess the binding of leukocytes to the V/I construct, VCAM-1 was blocked with an anti-VCAM-1 blocking antibody (3 μg of antibody/100 μL of medium; eBioscience, catalog no. 16-1061-85).

Flow Cytometry. Expression of the V/I construct was examined by immunolabeling with either an isotype control FITC-conjugated goat anti-rat IgG or a monoclonal anti-human ICAM-1 FITC-conjugated antibody. VCAM-1 expression was examined by immunolabeling with anti-mouse VCAM-1 followed by FITC-conjugated goat anti-rat IgG. The fluorescence was examined by flow cytometry using the BD Biosciences LSR II with analysis using Flow Jo version 7.2.1.

I-TASSER Molecular Modeling. The VCAM-1 cytoplasmic domain sequence was submitted to the I-TASSER program for automated protein structure and function prediction at the University of Michigan (Ann Arbor, MI).[32−34]

Statistics. Data were analyzed by one-way analysis of variance followed by Tukey's multiple-comparisons test or pairwise *t* tests (SigmaStat, Jandel Scientific).

RESULTS

The VCAM-1 Cytoplasmic Domain Is Highly Conserved among Species. Entrez-PubMed and Ensemble sequencing indicates that the 19-amino acid VCAM-1 cytoplasmic domain is highly conserved among mammalian species. There is 100% amino acid sequence identity among human, mouse, rabbit, rat, chimpanzee, rhesus macaque, Sumatran orangutan, common shrew, and microbat, with an only one- or two-conserved residue difference for dog, giant panda, horse, dolphin, pig, and African savanna elephant (Figure 1A). Recognizing the rarity of such species identity, we hypothesized that the VCAM-1 cytoplasmic domain was necessary for the VCAM-1 outside-in signaling cascade and VCAM-1-dependent leukocyte transendothelial migration.

Generation and Characterization of Endothelial Cells Stably Expressing VCAM-1/ICAM-1 (V/I) Chimeric Receptors. To gain mechanistic insight into the function of the VCAM-1 cytoplasmic domain, we used an established model endothelial cell line, mHEVa, for transfection with VCAM-1 mutation and deletion constructs. This endothelial cell line constitutively expresses VCAM-1 and is the simplest endothelial cell model for the examination of the functional outcomes of VCAM-1 signaling on leukocyte migration because it does not express other known adhesion molecules for leukocyte binding as determined by microarray and flow cytometry.[23] In other endothelial cells, analysis of VCAM-1 function during leukocyte migration is hampered because several adhesion molecules on endothelial cells mediate signaling upon binding leukocytes, thus complicating analysis. To engineer mutations in the cytoplasmic domain of VCAM-1 and distinguish these constructs from the endogenously expressed VCAM-1, we created a VCAM-1/ICAM-1 (V/I) chimeric receptor (Figure 1B), whereby the first two immunoglobulin-like domains of the chimera are replaced with the first two immunoglobulin-like domains of ICAM-1 and the remainder of the chimera consists of the third immunoglobulin-like domain through the carboxyl

A

Species (NCBI or Ensemble number)	cytoplasmic domain
Human (NCBI NP_542413.1):	RKANMKGSYSLVEAQKSKV
Mouse (NCBI CAA47989.1):	RKANMKGSYSLVEAQKSKV
Rabbit (NCBI NP_001075621.1):	RKANMKGSYSLVEAQKSKV
Rat (NCBI NP_037021.1):	RKANMKGSYSLVEAQKSKV
Chimpanzee (NCBI XP_001135527.2):	RKANMKGSYSLVEAQKSKV
Rhesus Macaque (NCBI XP_002801716.1):	RKANMKGSYSLVEAQKSKV
Sumatran Orangutan (NCBI NP_001126200.1):	RKANMKGSYSLVEAQKSKV
Common Shrew (Ensemble ENSSARP00000011070):	RKANMKGSYSLVEAQKSKV
Microbat (Ensemble ENSMLUP00000011488):	RKANMKGSYSLVEAQKSKV
Dog (NCBI NP_001003298.1):	R*R*ANMKGSYSLVEAQKSKV
Giant Panda (NCBI XP_002928199.1):	R*R*ANMKGSYSLVEAQKSKV
Horse (NCBI NP_001095120.1):	R*R*ANMKGSYSLVEAQKSKV
Dolphin (Ensemble ENSTTRP00000013710):	RKANMKGSYSLVEA*R*KSKV
Pig (NCBI NP_999056.1):	RKANM*R*GSYSLV*D*AQKSKV
African Savanna Elephant (NCBI XM_003409373.1):	R*R*ANMKGSY*D*LV*X*AQKSKV

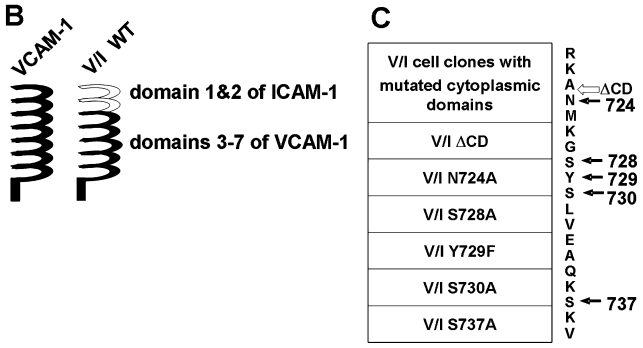


Figure 1. Schematic of the highly conserved 19-amino acid cytoplasmic domain of VCAM-1 and the VCAM-1/ICAM-1 (V/I) chimeric proteins. (A) The VCAM-1 19-amino acid cytoplasmic domain sequences for the species listed were obtained from Entrez-Pubmed and Ensemble. Conserved amino acids are underlined and in italics. (B) The V/I chimeric molecules were created by replacing the first two immunoglobulin-like domains of VCAM-1 with the first two immunoglobulin-like domains of ICAM-1. This chimeric receptor is designated V/I wild type (V/I WT). (C) The V/I chimeric receptor was mutated to delete the cytoplasmic domain or insert specific amino acid substitutions. The V/I receptor with the cytoplasmic domain deletion (V/I ΔCD) was created by inserting a "stop" codon after the alanine (A723) (white arrow). Alternatively, the VCAM-1 cytoplasmic domain serines, tyrosine, or asparagine was selected for a single-point mutation as indicated by the black arrows. The serines and asparagine were mutated to alanines, and the tyrosine was mutated to a phenylalanine. mHEV cells were transfected with plasmids containing these mutants and selected for stable expression. At least two separately derived clones were generated for each V/I construct. The V/I chimera in the mHEV clones was completely sequenced to ensure nucleotide sequence accuracy (data not shown).

cytoplasmic domain of VCAM-1. Thus, comparing the signals of endogenous wild type VCAM-1 to the signals of the V/I chimeric receptor with mutations is a useful model for studying the function of the cytoplasmic domain of VCAM-1. Expressing the V/I constructs in mHEVa cells limits the variables because the mHEVa cells do not express other adhesion molecules for leukocytes such as ICAM-1, PECAM-1, MAdCAM, etc. In the mHEVa endothelial cells expressing VCAM-1 and V/I chimeric receptors, VCAM-1 is stimulated by cross-linking with anti-VCAM-1 antibodies, and the V/I chimeric receptors are stimulated by cross-linking with a monoclonal anti-ICAM-1 antibody (84H10) that binds to domain 1 of ICAM-1. Cross-linking is initiated by addition of a secondary antibody.

To determine whether the VCAM-1 cytoplasmic domain is required for VCAM-1 function, we inserted a STOP codon after the first alanine (A723) within the cytoplasmic domain to create a VCAM-1 cytoplasmic domain deletion (V/I ΔCD)

(Figure 1C). We also hypothesized that the serines and tyrosine may regulate signaling, and therefore, we mutated amino acids S728, Y729, S730, and S737 in the cytoplasmic domain of the V/I WT construct (Figure 1C). To determine whether an amino acid other than serines or the tyrosine is necessary for VCAM-1 signaling, we targeted an uncharged amino acid within the cytoplasmic domain, N724 (Figure 1C); we hypothesized that this mutation would not affect VCAM-1 signaling. Each of the serines and the asparagine were mutated to alanine residues, and the tyrosine was mutated to a phenylalanine (Figure 1C). We expressed each of these constructs in the mHEVa cell line and created at least two separately derived clonal cell lines expressing each V/I construct (Figure 2B) as determined by immunolabeling with anti-ICAM-1 and flow cytometry. As demonstrated by immunolabeling with anti-ICAM-1 and flow cytometry, the mHEVa cell line does not express ICAM-1 (Figure 2B) as we previously reported.[23] In the cells expressing the mutant V/I chimeric receptors, there was no effect of the transfections on the endogenous surface expression of VCAM-1 as compared to that of nontransfected mHEV cells as determined by immunolabeling and flow cytometry (Figure 2A). To compare the molecular mass of the V/I chimeric receptors to V/I WT, VCAM-1 and V/I were immunoprecipitated from V/I WT cells and the V/I mutant cells (Figure 2C). The proteins were analyzed by Western blotting with anti-VCAM-1, stripped, and reprobed with anti-ICAM-1. The V/I WT receptors, V/I mutant receptors, and VCAM-1 are approximately the same molecular mass (Figure 2C).

Leukocyte Transendothelial Migration on VCAM-1 Involves the VCAM-1 Cytoplasmic Domain Amino Acids, S728, Y729, S730, and S737, but Not N724. We have reported that VCAM-1 signals are required for VCAM-1-dependent leukocyte migration. Therefore, we first determined whether the VCAM-1 cytoplasmic domain was necessary for VCAM-1-dependent leukocyte transendothelial migration in a parallel plate flow chamber with a 2 dyn/cm^2 laminar flow. Using blocking antibodies, we separately examined leukocyte migration on either the endogenous VCAM-1 or the V/I ΔCD receptors. For these studies, the V/I-transfected mHEV cells are the simplest endothelial cell model for the examination of leukocyte migration as a functional outcome of VCAM-1 signals because primary endothelial cells and other endothelial cell lines express many receptors that mediate leukocyte adhesion, including PECAM-1, VCAM-1, ICAM-1, CD99, selectins, etc. To examine VCAM-1-dependent leukocyte migration, the V/I-transfected mHEV cells were pretreated with saturating levels of adhesion blocking anti-ICAM-1 antibodies that bind ICAM-1 extracellular domain 1. To examine V/I-dependent leukocyte migration, the endothelial cells were pretreated with saturating levels of adhesion blocking anti-VCAM-1 antibodies. In addition, to block both VCAM-1- and V/I-dependent migration, endothelial cells were pretreated with both anti-VCAM-1 and anti-ICAM-1 blocking antibodies. Thus, under these conditions, we determined whether splenic lymphocyte (>90% lymphocytes) migration was supported by VCAM-1, the V/I WT chimeric receptor, or the V/I ΔCD chimeric receptor. In the V/I WT cell lines, blocking both VCAM-1 and the V/I WT chimeric receptor reduced the extent of migration by ∼70% as compared to the nontreated controls (Figure 3A); this reduction is similar to our previous report showing a 70% inhibition of lymphocyte migration by anti-VCAM-1 antibody treatment of mHEV cells that are not transfected with a V/I chimera.[12] Blocking either VCAM-1 or the V/I chimera in the V/I WT

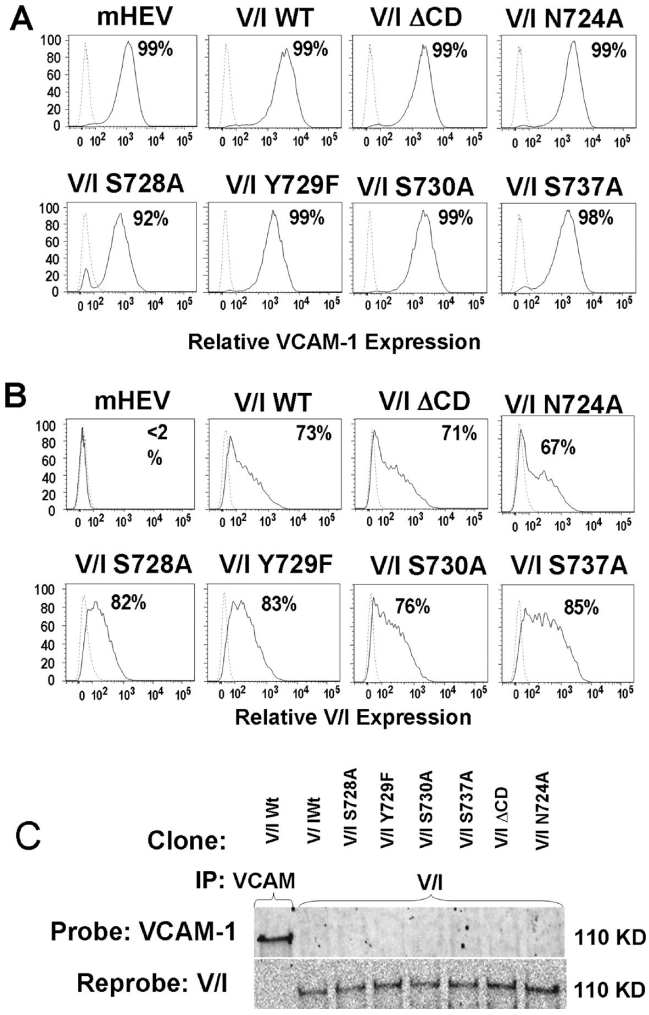


Figure 2. Expression of VCAM-1 and V/I receptors by the endothelial cells. (A) The mHEV clones with stable expression of V/I WT, V/I ΔCD, V/I N724A, V/I S728A, V/I Y729A, V/I S730A, or V/I S737A receptors were immunolabeled with anti-VCAM-1 primary antibodies (—) or isotype control antibodies (···) and FITC-conjugated anti-rat IgG secondary antibodies to determine surface VCAM-1 expression by flow cytometry. Shown is a representative graph of one of two separately derived clonal cell lines expressing each V/I chimeric receptor; the flow cytometry profile for the other clone of each chimera was similar (data not shown). (B) The clones expressing V/I WT and mutant V/I were immunolabeled with a FITC-conjugated anti-ICAM-1 antibody to determine V/I expression by flow cytometry. Shown is one representative graph of at least two separately derived clonal cell lines. Dotted lines are the isotype antibody controls. (C) The relative molecular masses of VCAM-1 and the V/I proteins were examined by Western blotting. VCAM-1 or V/I receptors were immunoprecipitated from the endothelial cell clones as indicated and separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The blots were probed with anti-VCAM-1. Then, the blots were stripped and reprobed with anti-ICAM-1 directed against domain 1 of ICAM-1 to detect the V/I proteins. The V/I WT or the V/I proteins with single-amino acid mutations or deletion of the short cytoplasmic domain were relatively the same overall molecular mass as VCAM-1 on SDS−PAGE when compared to the molecular mass standards.

cells resulted in an intermediate level of inhibition of lymphocyte migration (Figure 3A) as compared to that of the nontreated endothelium. There was no effect of isotype control antibodies as we previously published.[12]

dx.doi.org/10.1021/bi300925r | Biochemistry 2012, 51, 8235−8246

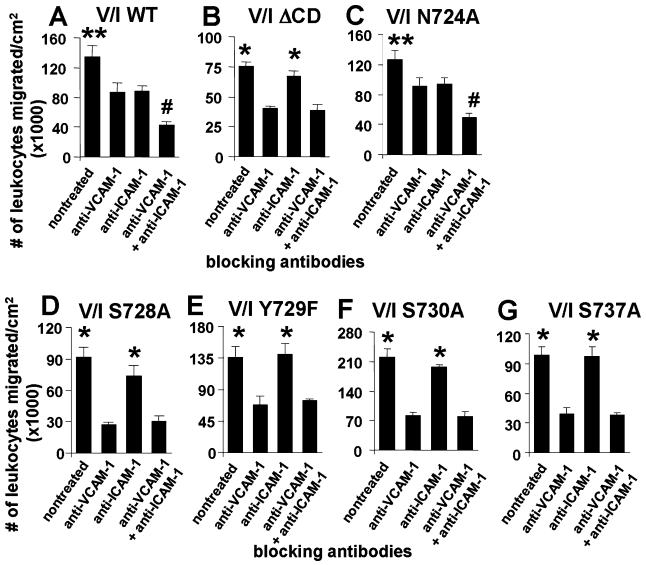


Figure 3. VCAM-1 cytoplasmic domain amino acids S728, Y729, S730, and S737, but not N724, are necessary for leukocyte transendothelial migration on VCAM-1. Leukocyte transendothelial migration across endothelial cells expressing (A) V/I WT, (B) V/I ΔCD, (C) V/I N724A, (D) V/I S728A, (E) V/I Y729A, (F) V/I S730A, or (G) V/I S737A was examined under laminar flow in a parallel plate flow chamber. The endothelial cells were pretreated for 15 min with the indicated blocking antibodies against VCAM-1, ICAM-1, or both. Secondary antibodies were not added to the cells, so the receptors were not activated by antibody cross-linking. Spleen leukocytes were added to each slide and allowed to settle for 5 min to initiate leukocyte−endothelial cell contact in the chamber as detailed in Materials and Methods. Then, for transendothelial migration, laminar flow was applied for 15 min at 2 dyn/cm^2. Migrated cells were identified as phase dark by phase contrast microscopy. Comparisons can be made only within an experiment because total migration can vary somewhat among experiments with the same cell line as previously reported.[12] Shown is one representative experiment of two or three. The other separately derived clone for each V/I chimera yielded similar results (data not shown). $N = 3$ slides per treatment. *$p < 0.05$ compared to the anti-VCAM-1-treated group and anti-VCAM-1- and anti-ICAM-1-treated group. **$p < 0.05$ compared to all groups. #$p < 0.05$ compared to the anti-VCAM-1-treated group and the anti-ICAM-1-treated group.

In contrast to the V/I WT chimeric receptor, the receptor with the cytoplasmic domain deletion, V/I ΔCD, did not support splenic lymphocyte transendothelial migration (Figure 3B). Briefly, blocking the V/I ΔCD receptor with anti-ICAM-1 did not inhibit lymphocyte transendothelial migration compared to that of the nontreated cells (Figure 3B), suggesting that the V/I ΔCD receptor did not contribute to lymphocyte migration. In contrast, blocking VCAM-1 resulted in a reduction in the extent of migration that was similar to blocking both VCAM-1 and the V/I ΔCD receptor, indicating that the lymphocytes had migrated using VCAM-1 and not the V/I ΔCD receptor (Figure 3B). Similar to the V/I ΔCD chimeric receptor (Figure 3B), V/I mutant receptors S728A, Y729F, S730A, and S737A did not contribute to leukocyte transendothelial migration across confluent monolayers of the endothelial cells (Figure 3D−G), indicating that these amino acids have a role in supporting leukocyte transendothelial migration.

To determine whether mutating another amino acid in the cytoplasmic domain affected leukocyte transendothelial migration, we used the V/I N724A construct. Similar to that of V/I WT (Figure 3A), antibody blocking of the V/I N724A receptor or the endogenous VCAM-1 inhibited migration compared to that of nontreated cells (Figure 3C). Moreover, blocking both VCAM-1 and the V/I N724A receptor inhibited migration more than blocking each receptor individually (Figure 3C). Therefore, the V/I N724A mutation did not affect leukocyte transendothelial migration. Thus, S728, Y729, S730, and S737, but not N724, are necessary for leukocyte transendothelial migration.

Transfection with V/I Mutants Does Not Alter Leukocyte Adhesion. We examined endothelial cell−leukocyte adhesion to the V/I ΔCD receptor or V/I S728A, V/I Y729F, V/I S730A, and V/I S737A receptors. Similar to our previous reports with mHEV cells not transfected with a V/I chimera,[12] anti-VCAM-1 resulted in a 65−75% inhibition of lymphocyte adhesion (Figure 4). The mutations did not reduce the level of

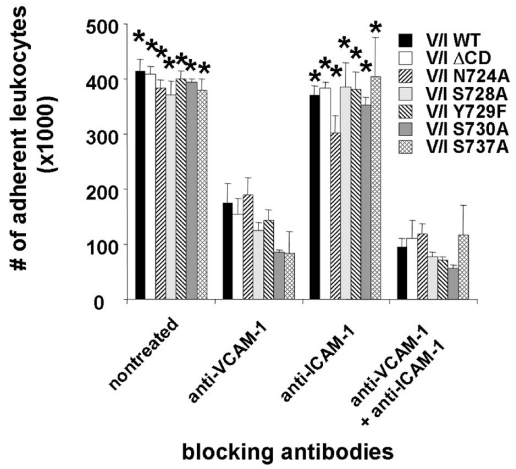


Figure 4. Transfection with V/I mutants does not alter leukocyte adhesion. Leukocyte binding to the endothelial cells expressing V/I WT, V/I ΔCD, V/I N724A, V/I S728A, V/I Y729A, V/I S730A, or V/I S737A was assessed at 5 min using an adhesion assay. Shown is the mean ± the standard error of the mean from three to four experiments. The other separately derived clone for each V/I chimera had similar results (data not shown). In each experiment, an average was obtained from triplicate wells. *$p < 0.05$ compared to the anti-VCAM-1-treated group and the anti-VCAM-1- and anti-ICAM-1-treated group.

adhesion (Figure 4). Although there was minimal inhibition of total adhesion with anti-ICAM-1 for the initial adhesion of spleen lymphocytes to the clones (Figure 4), the V/I WT receptor but not the V/I serine/tyrosine mutant receptors significantly contributed to the lymphocyte transendothelial migration (Figure 3). Thus, the V/I WT receptor expression levels were sufficiently high to contribute to lymphocyte migration.

VCAM-1 Activation of Rac1 Utilizes VCAM-1 Cytoplasmic Domain Amino Acids S730 and S737, but Not S728, Y729, or N724. We and others have previously reported that antibody cross-linking of VCAM-1 rapidly activates Rac1.[15,21] The signals downstream of Rac1 are required for VCAM-1-dependent leukocyte transendothelial migration.[24,26,35] Therefore, we determined whether the cytoplasmic domain of VCAM-1 is required for VCAM-1 activation of Rac1 using the cells expressing V/I WT or V/I mutants. Cross-linking the V/I WT chimeric receptors with an anti-ICAM-1−secondary antibody complex significantly activated Rac1 as

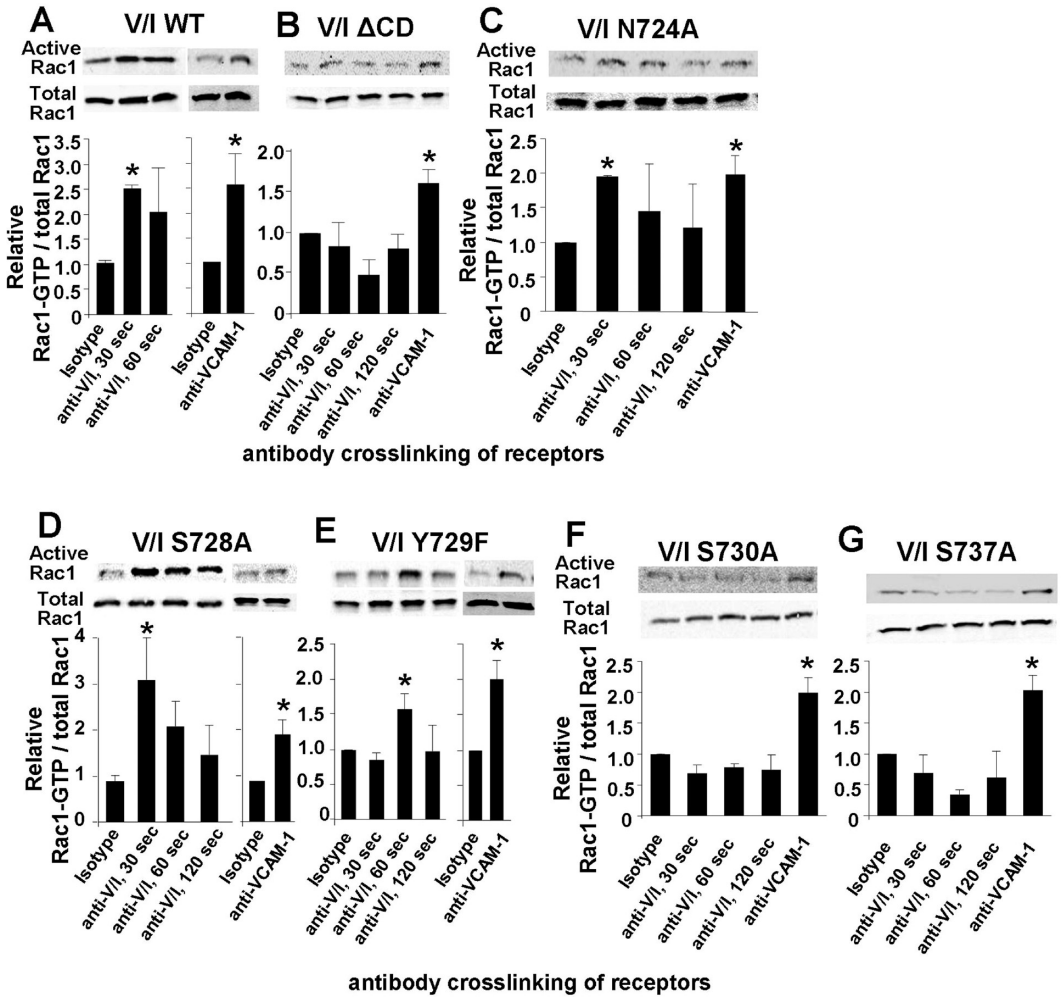


Figure 5. VCAM-1 cytoplasmic domain amino acids S730 and S737, but not S728, Y729, or N724, are necessary for Rac1 signaling. To cross-link and activate the V/I chimeric receptors, anti-ICAM-1, which is specific for domain 1 of ICAM-1, was incubated with a secondary antibody for 5 min and then added to endothelial cells expressing (A) V/I WT, (B) V/I ΔCD, (C) V/I N724A, (D) V/I S728A, (E) V/I Y729A, (F) V/I S730A, or (G) V/I S737A for 30, 60, and 120 s. Then, Rac1 activity was determined. As a positive control, an anti-VCAM-1 and a secondary antibody were added to the cells to cross-link and activate the endogenous VCAM-1 for 30 or 60 s. $N = 2-4$ experiments for each V/I chimeric receptor. The blots are representative blots. The data in the graphs are the average of the two separately derived clones for each V/I chimeric receptor. $^*p < 0.05$ compared to the isotype antibody control.

compared to the treatment of the cells with an isotype control antibody−secondary antibody complex (Figure 5A). Activation of these cells by cross-linking VCAM-1 with an anti-VCAM-1−secondary antibody complex activated Rac1 (Figure 5A). This 1.5−2-fold increase in Rac1 activity in 30−60 s is similar to the results in our previous report with anti-VCAM-1 activation of Rac1 in mHEV cells not expressing V/I chimeric receptors.[15] In contrast, when the V/I ΔCD receptors were cross-linked with antibodies, Rac1 was not activated, indicating that the VCAM-1 cytoplasmic domain is necessary for VCAM-1 activation of Rac1 (Figure 5B). As a positive control, Rac1 was activated by antibody cross-linking of the endogenous VCAM-1 on the V/I ΔCD cells (Figure 5B), indicating that the loss of V/I ΔCD receptor activation of Rac1 was due to the loss of the V/I construct cytoplasmic domain and not to the inability of the endothelial cell lines to activate Rac1.

Antibody cross-linking of the V/I N724A, V/I S728A, or V/I Y729F receptor activated Rac1 (Figure 5C−E), suggesting that these three amino acids were not necessary for initiating VCAM-1 cytoplasmic domain activation of Rac1. The positive control, antibody cross-linking of the endogenous VCAM-1, activated Rac1 in the cells expressing V/I N724A, V/I S728A, or V/I Y729F (Figure 5C−E). In contrast to these amino acids, antibody cross-linking of the V/I S730A receptor or the V/I S737A receptor did not activate Rac1 (Figure 5F,G), demonstrating that these two amino acids are necessary for VCAM-1 activation of Rac1. As a positive control, Rac1 was activated by antibody cross-linking of the endogenous VCAM-1 on the cells expressing V/I S730A or V/I S737A (Figure 5F,G), suggesting that transfection has not induced an inherent change within these endothelial cell lines that prevents the cells from activating Rac1. Thus, S730 and S737, but not S728, Y729, or N724, are involved in VCAM-1 activation of Rac1.

The Cytoplasmic Domain Amino Acids for VCAM-1 Activation of Calcium Fluxes Are at Sites Distinct from Those for VCAM-1 Activation of Rac1. We and others have previously reported that antibody cross-linking of VCAM-1 rapidly activates calcium fluxes.[15,21] The calcium fluxes are required for VCAM-1 activation of NADPH oxidase, which is necessary for leukocyte recruitment in vitro and in vivo.[7,26,36,37] Therefore, we determined whether the cytoplasmic domain of VCAM-1 is required for VCAM-1 activation of calcium fluxes

dx.doi.org/10.1021/bi300925r | *Biochemistry* 2012, 51, 8235−8246

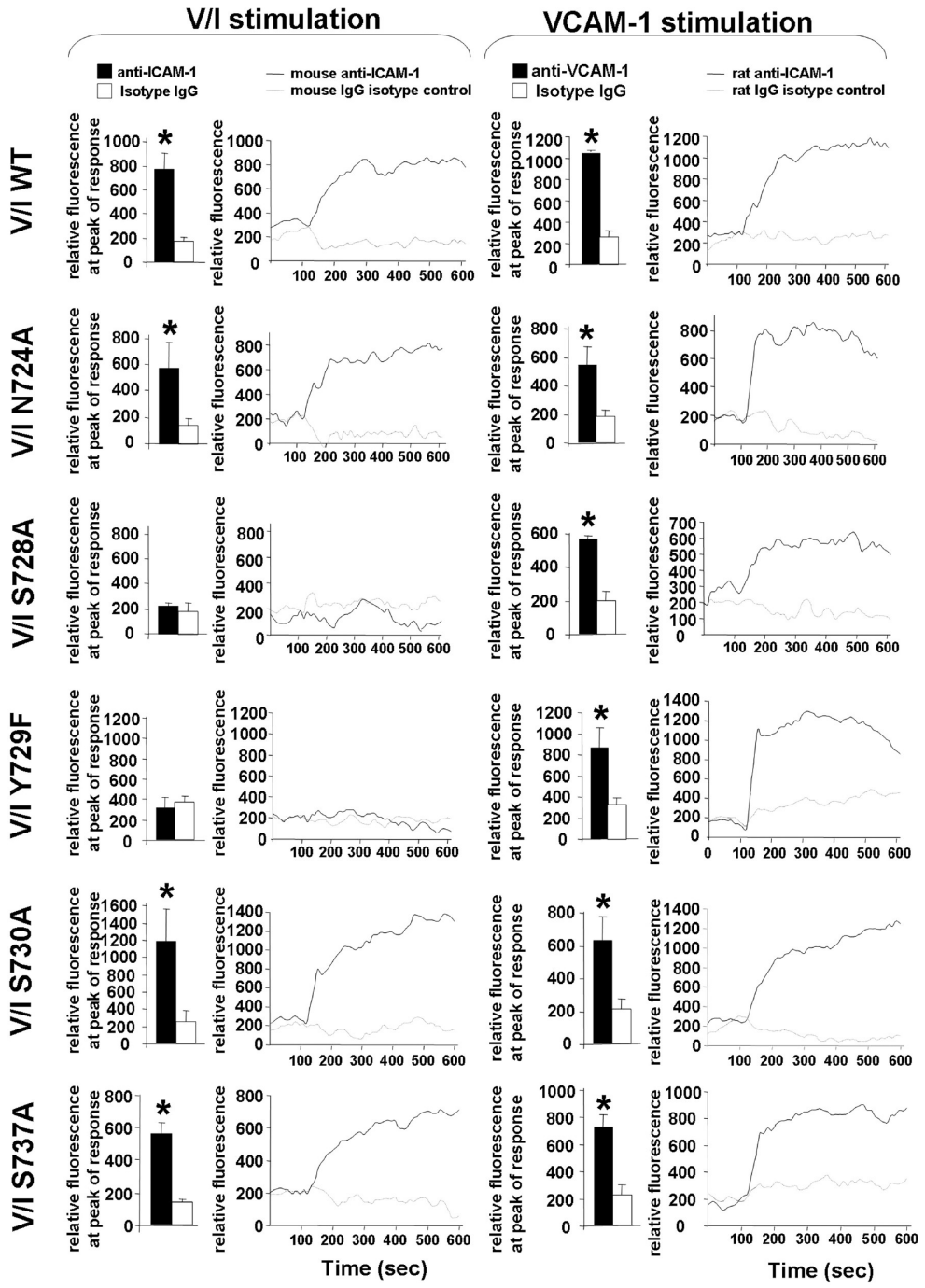


Figure 6. VCAM-1 cytoplasmic domain amino acids S730 and S737, but not S728, Y729, or N724, are necessary for calcium fluxes. Endothelial cells expressing V/I WT, V/I ΔCD, V/I N724A, V/I S728A, V/I Y729A, V/I S730A, or V/I S737A were loaded with fluo-4 and examined for V/I- or VCAM-1-induced calcium fluxes. To cross-link and activate the V/I chimeras, anti-ICAM-1, which is specific for domain 1 of ICAM-1, was incubated with a secondary antibody for 5 min and then added to fluo-4-loaded endothelial cells. Then, the relative fluo-4 fluorescence was examined. As a positive control, an anti-VCAM-1 with a secondary antibody was added to the cells to cross-link and activate the endogenous VCAM-1 for 30 or 60 s. Shown are representative calcium fluxes. Also, data from triplicate experiments are presented as the magnitude of the calcium response (peak height; mean ± the standard error of the mean). The other separately derived clone for each V/I chimera produced similar results (data not shown). *p < 0.05 compared to the isotype control.

using the cells expressing V/I WT or V/I mutants. Cross-linking the V/I WT receptors with an anti-ICAM-1−secondary antibody complex significantly activated a calcium flux as compared to the treatment of the cells with an isotype control (Figure 6). Activation of these cells by cross-linking VCAM-1 with an anti-VCAM-1−secondary antibody complex activated a calcium flux (Figure 6). This rapid activation of a calcium flux by anti-VCAM-1 is similar to our previous report with anti-VCAM-1 activation of calcium in mHEV cells not expressing the V/I chimera.[15] In contrast, when we cross-linked the V/I S728A receptor or the V/I Y729A receptor, each failed to activate a calcium response (Figure 6), demonstrating that these two amino acids are necessary for VCAM-1 activation of calcium fluxes. As a positive control, calcium fluxes were activated by

dx.doi.org/10.1021/bi300925r | *Biochemistry* 2012, 51, 8235−8246

antibody cross-linking of the endogenous VCAM-1 on the cells expressing the V/I S728A receptor or the V/I Y729A receptor (Figure 6), suggesting that transfection has not induced an inherent change within these endothelial cell lines that prevents the cells from activating a calcium flux.

Antibody cross-linking of the V/I N724A, V/I S730A, or V/I S737A receptor activated calcium flux (Figure 6), suggesting that these three amino acids were not necessary for initiating VCAM-1 cytoplasmic domain activation of calcium fluxes. The positive control, antibody cross-linking of the endogenous VCAM-1, activated a calcium flux in the cells expressing V/I N724A, V/I S728A, or V/I Y729F (Figure 6). Thus, S728 and Y729, but not S730, S737, or N724, were involved in VCAM-1 activation of calcium fluxes, suggesting that S728 and Y729 are at a distinct functional site than that of S730 and S737 on the cytoplasmic domain of VCAM-1.

Molecular Model of the VCAM-1 Cytoplasmic Domain. The structure of the cytoplasmic domain of VCAM-1 is not known. Therefore, the I-TASSER molecular modeling program[32−34] was used to model the VCAM-1 cytoplasmic domain. All predictions from the I-TASSER modeling for the VCAM-1 cytoplasmic domain were α-helical. The VCAM-1 model with the best I-TASSER confidence score is shown from three views rotated on the vertical axis in Figure 7A. There is a horizontal plane containing S728 and Y729 (Figure 7A, right panel). Interestingly, on the other side of the helix, S730 and S737 are on a vertical plane in the helix (Figure 7A, left panel), suggesting that S730 and S737, and S728 and Y729, may have different functions in VCAM-1 signaling. This modeling is consistent with the data in Figures 5 and 6 demonstrating that S730 and S737 are required for Rac1 signaling but S728 and Y729 are required for calcium fluxes. S728 and Y729, and S730 and S737, were necessary for VCAM-1-dependent leukocyte transendothelial migration, because these amino acids activate calcium fluxes and Rac1, respectively, and calcium and Rac1 are each required for VCAM-1 activation of NOX2 during leukocyte transendothelial migration (Figure 7B).[7]

DISCUSSION

In this study, we demonstrate, for the first time, that the VCAM-1 cytoplasmic domain is necessary for VCAM-1-stimulated endothelial cell signaling and leukocyte transendothelial migration. We also demonstrate that two amino acids within the VCAM-1 cytoplasmic domain, S730 and S737, are necessary for VCAM-1 activation of the downstream signal Rac1 and for leukocyte transmigration. In contrast, S728 and Y729 in the VCAM-1 cytoplasmic domain were not necessary for activation of Rac1 but were required for VCAM-1 activation of calcium fluxes and leukocyte transendothelial migration. Mutation of another nearby amino acid, N724 in the VCAM-1 cytoplasmic domain, did not affect Rac1 activation, calcium fluxes, or leukocyte transendothelial migration, indicating that the loss of leukocyte migration with a mutation of VCAM-1 S728, Y729, S730, or S737 was not a general effect of mutating the cytoplasmic domain. Furthermore, modeling of the VCAM-1 cytoplasmic domain indicates that the cytoplasmic domain is an α-helix with amino acids S728 and Y729 located in a plane on one side of the helix and S730 and S737 located in a plane on the opposite side of the α-helix. The opposing locations on the α-helix for these two sets of amino acids suggest that these amino acids have distinct regulatory functions. This model is consistent with our findings that S730 and S737 function in the activation of Rac1 for promotion of leukocyte transendothelial

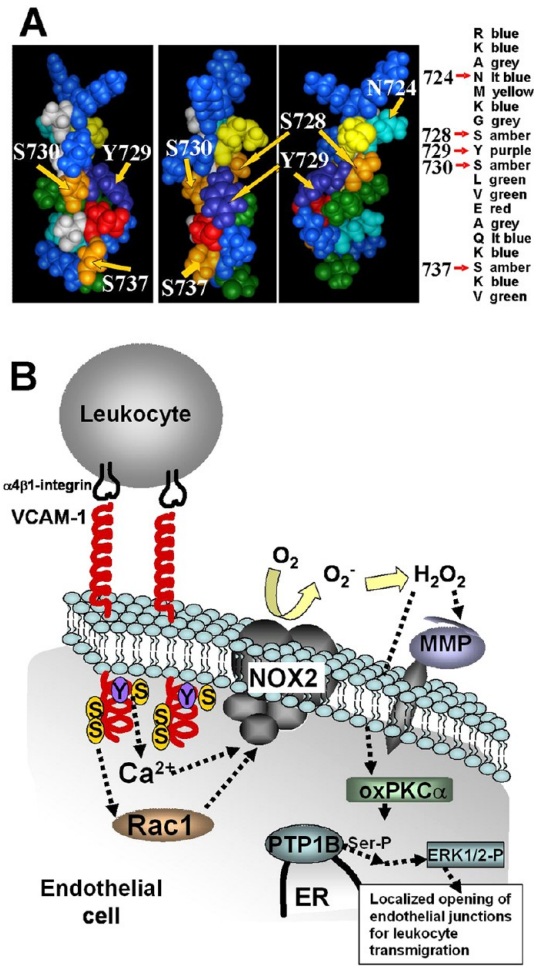


Figure 7. Model of the VCAM-1 cytoplasmic domain structure and function. (A) The VCAM-1 cytoplasmic domain 19-amino acid sequence (RKANMKGSYSLVEAQKSKV) was submitted to the I-TASSER database for structural prediction. Shown is the model with the highest predictive accuracy score. The three images show three different side views rotated on the vertical axis of the VCAM-1 cytoplasmic domain with the membrane proximal region at the top and the carboxyl terminus at the bottom of the image. The serines, tyrosine, and asparagine are indicated. The I-TASSER program color-coded the amino acids as follows: blue for basic amino acids R and K, gray for A and G, light (lt) blue for polar and uncharged amino acids N and Q, yellow for M, purple for Y, red for acidic amino acid E, amber for S, and green for nonpolar amino acids L and V. S728 and Y729 are on a horizontal plane, and on the opposite side of the helix are located S730 and S737 in a vertical plane. (B) Schematic for VCAM-1 signaling. Upon antibody cross-linking of VCAM-1, VCAM-1 S728 and Y729 function in the activation of calcium fluxes and VCAM-1 S730 and S737 function in the activation of Rac1. The calcium and Rac1 then activate endothelial cell NOX2. NOX2 catalyzes the production of superoxide that then dismutates to H_2O_2. VCAM-1 induces the production of only 1 μM H_2O_2. H_2O_2 activates endothelial cell-associated matrix metalloproteinases (MMPs) that degrade extracellular matrix and endothelial cell surface receptors in cell junctions. H_2O_2 also diffuses through membranes to oxidize and transiently activates endothelial cell protein kinase Cα (PKCα). PKCα phosphorylates and activates protein tyrosine phosphatase 1B (PTP1B) on the endoplasmic reticulum (ER). PTP1B is not oxidized. PTP1B activates signals that induce phosphorylation and activation of ERK1/2. These signals through reactive oxygen species (ROS), MMPs, PKCα, PTP1B, and ERK1/2 are required for VCAM-1-dependent leukocyte transendothelial migration.

dx.doi.org/10.1021/bi300925r | Biochemistry 2012, 51, 8235−8246

migration and that S728 and Y729 function in the activation of calcium fluxes in the regulation of leukocyte transendothelial migration.

S728 and Y729, and S730 and S737, are distinct sites in the VCAM-1 cytoplasmic domain for signaling; S728 and Y729 mediate the activation of calcium fluxes, and S730 and S737 mediate the activation of Rac1. Calcium and Rac1 function in the translocation of the cytoplasmic subunits of NOX2 to the membrane and activation of the active complex (Figure 7B) in several cell types.[38−45] NOX2 is then required for VCAM-1-dependent activation of PKCα, PTP1B, ERK1/2, and VCAM-1-dependent leukocyte transendothelial migration in vitro and in vivo (Figure 7B).[7,22,24,35]

Although functions for the cytoplasmic domain of VCAM-1 have not been previously reported, the functions of the cytoplasmic domain of other members of the immunoglobulin superfamily have been studied. Studies of other immunoglobulin superfamily molecules have indicated that cytoplasmic domain phosphorylation regulates signaling, membrane localization, and recruitment of protein to the cytoplasmic domain.[46−52] Several immunoglobulin superfamily members, including the T cell receptor, B cell receptor, platelet−endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), and B and T lymphocyte attenuator (BTLA), signal through regulation of tyrosine phosphorylation in their immunoreceptor tyrosine-based inhibitory motif (ITIM) or immunoreceptor tyrosine-based activating motif (ITAM). The ITIMs in the cytoplasmic domain of the immunoglobulin superfamily proteins PECAM-1 and BTLA are necessary for initiating a downstream signaling cascade.[48,53] In contrast, ITIM and ITAM are not present in the VCAM-1 cytoplasmic domain, suggesting alternative mechanisms for VCAM-1 signaling. In addition to ITIM regulation of PECAM-1, the PECAM-1 cytoplasmic domain is regulated by serine phosphorylation[54] and by palmitoylation of cysteine 595 in its cytoplasmic domain.[55] In contrast, the VCAM-1 cytoplasmic domain is not regulated by cysteine palmitoylation because it does not contain a cysteine. PECAM-1 is also reported to contain a 20-amino acid α-helix within the 113-amino acid PECAM-1 cytoplasmic domain. This PECAM-1 cytoplasmic domain α-helix forms only upon interaction with the membrane lipid environment.[56] We also report a VCAM-1 model with an α-helix in the VCAM-1 cytoplasmic domain. However, there is no sequence homology between the PECAM-1 and VCAM-1 helix, suggesting that there are likely different functions for the helix in these receptors.

The cytoplasmic domain of the adhesion molecule ICAM-1 is necessary for ICAM-1 signaling. Interestingly, two studies examining ICAM-1 in mouse and rat brain endothelium showed that deleting the whole ICAM-1 cytoplasmic domain drastically reduced the level of T cell transendothelial migration and, upon ICAM-1 cross-linking, reduced the level of activation of RhoGTPase, a downstream mediator of ICAM-1 signaling.[57−59] ICAM-1 contains a tyrosine within an ITIM at amino acids 480−488.[60] However, a mutation of the ICAM-1 cytoplasmic tyrosine to a phenylalanine did not affect the downstream activation of the RhoGTPase but did partially block migration.[57] This lack of an effect on RhoGTPase with the mutated ICAM-1 tyrosine is similar to our data demonstrating that mutation of VCAM-1 Y729 did not alter activation of Rac1. However, VCAM-1 Y729 mutation blocks calcium fluxes and leukocyte transendothelial migration. In contrast, unlike the ICAM-1 cytoplasmic domain, which does not contain serines and does not have an amino acid(s) identified for activation of RhoGTPase, we demonstrated that S730 and S737 are necessary for VCAM-1 activation of Rac1. It is also reported that the ICAM-1 membrane proximal RKIKK sequence is required for the association of ICAM-1 with cytoskeletal proteins and is required for leukocyte transendothelial migration.[61] An RKIKK sequence is not present in the cytoplasmic domain of VCAM-1, and the ICAM-1 cytoplasmic domain sequence does not have amino acid sequence homology with the VCAM-1 cytoplasmic domain.[57,58]

Several vascular adhesion molecules that are members of the immunoglobulin superfamily have some amino acid homology among species. For PECAM-1, the mouse and human 113-amino acid cytoplasmic domain sequence is ∼70% identical, and within this domain, the longest sequence of identity consists of eight amino acids. For ICAM-1, the mouse and human 27-amino acid cytoplasmic domain sequence is ∼50% identical, and within this domain, the longest sequence of identity consists of the five membrane proximal amino acids.[57,58] In contrast, the entire 19-amino acid cytoplasmic domain of VCAM-1 is 100% identical in amino acid sequence among many mammals, including human, mouse, rabbit, rat, chimpanzee, rhesus macaque, Sumatran orangutan, common shrew, and microbat. This 100% amino acid identity observed for the VCAM-1 cytoplasmic domain among species is rare, suggesting a critical role for this domain in function and evolution.

In summary, we have demonstrated that the serines and the tyrosine within the VCAM-1 cytoplasmic domain form distinct sites that are necessary for VCAM-1-stimulated endothelial cell signaling and leukocyte transendothelial migration. VCAM-1 S730 and S737 were required for activation of Rac1 and leukocyte transendothelial migration but not calcium fluxes. VCAM-1 S728 and Y729 were not required for activation of Rac1 but were required for calcium fluxes and leukocyte transendothelial migration. Calcium and Rac1 function together to activate NOX2, and then NOX2 is required for leukocyte transendothelial migration in vitro and in vivo (Figure 7B).[26,36] These results define a functional role for the cytoplasmic domain of VCAM-1, whereby, in addition to the extracellular domains being involved in leukocyte-endothelial cell binding, there are distinct sites within the cytoplasmic domain of VCAM-1 that actively regulate VCAM-1 signaling during leukocyte recruitment.

AUTHOR INFORMATION

Corresponding Author

*Division of Allergy-Immunology, Northwestern University Feinberg School of Medicine, McGaw M304, 240 E. Huron, Chicago, IL 60611. Telephone: (312) 503-0906. Fax: (312) 503-0078. E-mail: j-cook-mills@northwestern.edu.

Funding

These studies were supported by National Institutes of Health Grants R01 HL69428 (J.M.C.-M.) and R01 AT004837 (J.M.C.-M.) and American Heart Association Grants 0855583G and 12GRNT12100020 (J.M.C.-M.).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

calcein-AM, calcein acetoxymethyl ester; ERK1/2, extracellular regulated kinases 1 and 2; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule-1; IgG, immunoglobulin G; ITAM, immunoreceptor tyrosine-based activating

motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAdCAM, mucosal addressin cell adhesion molecule; MCP-1, monocyte chemotactic protein-1; MMPs, matrix metalloproteinases; PECAM-1, platelet−endothelial cell adhesion molecule-1; PKCα, protein kinase Cα; PTP1B, protein tyrosine phosphatase 1B; VCAM-1, vascular cell adhesion molecule-1; V/I, VCAM-1 with the first two immunoglobulin-like domains replaced with the first two immunoglobulin-like domains of ICAM-1; V/I ΔCD, V/I construct without the cytoplasmic domain.

REFERENCES

(1) Alon, R., Kassner, P. D., Carr, M. W., Finger, E. B., Hemler, M. E., and Springer, T. A. (1995) The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J. Cell Biol. 128*, 1243−1253.

(2) Chan, B. M., Elices, M. J., Murphy, E., and Hemler, M. E. (1992) Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of $\alpha4\beta1$ (VLA-4) and $\alpha4\beta7$ on the human B cell line JY. *J. Biol. Chem. 267*, 8366−8370.

(3) Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E., and Lobb, R. R. (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell 60*, 577−584.

(4) Abonia, J. P., Hallgren, J., Jones, T., Shi, T., Xu, Y., Koni, P., Flavell, R. A., Boyce, J. A., Austen, K. F., and Gurish, M. F. (2006) α-4 integrins and VCAM-1, but not MAdCAM-1, are essential for recruitment of mast cell progenitors to the inflamed lung. *Blood 108*, 1588−1594.

(5) Bochner, B. S., Luscinskas, F. W., Gimbrone, M. A., Jr., Newman, W., Sterbinsky, S. A., Derse-Anthony, C. P., Klunk, D., and Schleimer, R. P. (1991) Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: Contributions of endothelial cell adhesion molecules. *J. Exp. Med. 173*, 1553−1557.

(6) Miyake, K., Medina, K., Ishihara, K., Kimoto, M., Auerbach, R., and Kincade, P. W. (1991) A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J. Cell Biol. 114*, 557−565.

(7) Cook-Mills, J. M., Marchese, M., and Abdala-Valencia, H. (2011) Vascular Cell Adhesion Molecule-1 Expression and Signaling during Disease: Regulation by Reactive Oxygen Species and Antioxidants. *Antioxid. Redox Signaling 15*, 1607−1638.

(8) Chin, J. E., Hatfield, C. A., Winterrowd, G. E., Brashler, J. R., Vonderfecht, S. L., Fidler, S. F., Griffin, R. L., Kolbasa, K. P., Krzesicki, R. F., Sly, L. M., Staite, N. D., and Richards, I. M. (1997) Airway recruitment of leukocytes in mice is dependent on α4-integrins and vascular cell adhesion molecule-1. *Am. J. Physiol. 272*, L219−L229.

(9) Allavena, R., Noy, S., Andrews, M., and Pullen, N. (2010) CNS Elevation of Vascular and Not Mucosal Addressin Cell Adhesion Molecules in Patients with Multiple Sclerosis. *Am. J. Pathol. 176*, 556−562.

(10) Iiyama, K., Hajra, L., Iiyama, M., Li, H., DiChiara, M., Medoff, B. D., and Cybulsky, M. I. (1999) Patterns of Vascular Cell Adhesion Molecule-1 and Intercellular Adhesion Molecule-1 Expression in Rabbit and Mouse Atherosclerotic Lesions and at Sites Predisposed to Lesion Formation. *Circ. Res. 85*, 199−207.

(11) Gurtner, G. C., Davis, V., Li, H., McCoy, M. J., Sharpe, A., and Cybulsky, M. I. (1995) Targeted disruption of the murine VCAM1 gene: Essential role of VCAM-1 in chorioallantoic fusion and placentation. *Genes Dev. 9*, 1−14.

(12) Matheny, H. E., Deem, T. L., and Cook-Mills, J. M. (2000) Lymphocyte migration through monolayers of endothelial cell lines involves VCAM-1 signaling via endothelial cell NADPH oxidase. *J. Immunol. 164*, 6550−6559.

(13) Tudor, K.-S. R. S., Hess, K. L., and Cook-Mills, J. M. (2001) Cytokines modulate endothelial cell intracellular signal transduction required for VCAM-1-dependent lymphocyte transendothelial migration. *Cytokine 15*, 196−211.

(14) Deem, T. L., and Cook-Mills, J. M. (2004) Vascular cell adhesion molecule 1 (VCAM-1) activation of endothelial cell matrix metalloproteinases: Role of reactive oxygen species. *Blood 104*, 2385−2393.

(15) Cook-Mills, J. M., Johnson, J. D., Deem, T. L., Ochi, A., Wang, L., and Zheng, Y. (2004) Calcium mobilization and Rac1 activation are required for VCAM-1 (vascular cell adhesion molecule-1) stimulation of NADPH oxidase activity. *Biochem. J. 378*, 539−547.

(16) Cook-Mills, J. M. (2006) Hydrogen peroxide activation of endothelial cell-associated MMPs during VCAM-1-dependent leukocyte migration. *Cell. Mol. Biol. (Oxford) 52*, 8−16.

(17) Abdala-Valencia, H., and Cook-Mills, J. M. (2006) VCAM-1 signals activate endothelial cell protein kinase Cα via oxidation. *J. Immunol. 177*, 6379−6387.

(18) Deem, T. L., Abdala-Valencia, H., and Cook-Mills, J. M. (2007) VCAM-1 activation of endothelial cell protein tyrosine phosphatase 1B. *J. Immunol. 178*, 3865−3873.

(19) van Wetering, S., van den Berk, N., van Buul, J. D., Mul, F. P., Lommerse, I., Mous, R., ten Klooster, J. P., Zwaginga, J. J., and Hordijk, P. L. (2003) VCAM-1-mediated Rac signaling controls endothelial cell-cell contacts and leukocyte transmigration. *Am. J. Physiol. 285*, C343−C352.

(20) Cook-Mills, J. M. (2002) Reactive oxygen species regulation of immune function. *Mol. Immunol. 39*, 497−498.

(21) van Wetering, S., van den Berk, N., van Buul, J. D., Mul, F. P. J., Lommerse, I., Mous, R., Klooster, J.-P. t., Zwaginga, J.-J., and Hordijk, P. L. (2003) VCAM-1-mediated Rac signaling controls endothelial cell-cell contacts and leukocyte transmigration. *Am. J. Physiol. 285*, C343−C352.

(22) Abdala-Valencia, H., Berdnikovs, S., and Cook-Mills, J. M. (2011) Mechanisms for vascular cell adhesion molecule-1 activation of ERK1/2 during leukocyte transendothelial migration. *PLoS One 6*, e26706.

(23) Cook-Mills, J. M., Gallagher, J. S., and Feldbush, T. L. (1996) Isolation and characterization of high endothelial cell lines derived from mouse lymph nodes. *In Vitro Cell. Dev. Biol.: Anim. 32*, 167−177.

(24) Deem, T. L., Abdala-Valencia, H., and Cook-Mills, J. M. (2007) VCAM-1 Activation of PTP1B in Endothelial Cells. *J. Immunol. 178*, 3865−3873.

(25) Cook-Mills, J. M., Gallagher, J. S., and Feldbush, T. L. (1996) Isolation and characterization of high endothelial cell lines derived from mouse lymph nodes. *In Vitro Cell. Dev. Biol.: Anim. 32*, 167−177.

(26) Matheny, H. E., Deem, T. L., and Cook-Mills, J. M. (2000) Lymphocyte Migration through Monolayers of Endothelial Cell Lines involves VCAM-1 Signaling via Endothelial Cell NADPH Oxidase. *J. Immunol. 164*, 6550−6559.

(27) Qureshi, M. H., Cook-Mills, J., Doherty, D. E., and Garvy, B. A. (2003) TNF-α-Dependent ICAM-1- and VCAM-1-Mediated Inflammatory Responses Are Delayed in Neonatal Mice Infected with *Pneumocystis carinii*. *J. Immunol. 171*, 4700−4707.

(28) Nobis, U., Pries, A. R., Cokelet, G. R., and Gaehtgens, P. (1985) Radial distribution of white cells during blood flow in small tubes. *Microvasc. Res. 29*, 295−304.

(29) Smith, M. L., Long, D. S., Damiano, E. R., and Ley, K. (2003) Near-wall micro-PIV reveals a hydrodynamically relevant endothelial surface layer in venules in vivo. *Biophys. J. 85*, 637−645.

(30) Lipowsky, H. H. (2005) Microvascular rheology and hemodynamics. *Microcirculation 12*, 5−15.

(31) Ager, A., and Mistry, S. (1988) Interaction between lymphocytes and cultured high endothelial cells: An *in vitro* model of lymphocyte migration across high endothelial venule endothelium. *Eur. J. Immunol. 18*, 1265−1274.

(32) Zhang, Y. (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinf. 9*, 40.

(33) Zhang, Y. (2009) I-TASSER: Fully automated protein structure prediction in CASP8. *Proteins: Struct., Funct., Bioinf. 77*, 100−113.

(34) Roy, A., Kucukural, A., and Zhang, Y. (2010) I-TASSER: A unified platform for automated protein structure and function prediction. *Nat. Protoc. 5*, 725−738.

(35) Abdala-Valencia, H., and Cook-Mills, J. M. (2006) VCAM-1 Signals Activate Endothelial Cell Protein Kinase Ca Via Oxidation. *J. Immunol. 177*, 6379−6387.
(36) Abdala-Valencia, H., Earwood, J., Bansal, S., Jansen, M., Babcock, G., Garvy, B., Wills-Karp, M., and Cook-Mills, J. M. (2007) Nonhematopoietic NADPH oxidase regulation of lung eosinophilia and airway hyperresponsiveness in experimentally induced asthma. *Am. J. Physiol. 292*, L1111−L1125.
(37) Cook-Mills, J. M., Johnson, J. D., Deem, T. L., Ochi, A., Wang, L., and Zheng, Y. (2004) Calcium mobilization and Rac1 activation are required for VCAM-1 (vascular cell adhesion molecule-1) stimulation of NADPH oxidase activity. *Biochem. J. 378*, 539−547.
(38) Lang, M. L., and Kerr, M. A. (2000) Characterization of FcαR-triggered Ca^{2+} signals: Role in neutrophil NADPH oxidase activation. *Biochem. Biophys. Res. Commun. 276*, 749−755.
(39) McPhail, L. C., Clayton, C. C., and Snyderman, R. (1984) The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. *J. Biol. Chem. 259*, 5768−5775.
(40) Movitz, C., Sjolin, C., and Dahlgren, C. (1997) A rise in ionized calcium activates the neutrophil NADPH-oxidase but is not sufficient to directly translocate cytosolic p47phox or p67phox to b cytochrome containing membranes. *Inflammation 21*, 531−540.
(41) Zhou, H., Duncan, R. F., Robison, T. W., Gao, L., and Forman, H. J. (1997) Ca^{2+}-dependent p47phox translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. *Am. J. Physiol. 273*, L1042−L1047.
(42) Goodman, E. B., and Tenner, A. J. (1992) Signal transduction mechanisms of C1q-mediated superoxide production. Evidence for the involvement of temporally distinct staurosporine-insensitive and sensitive pathways. *J. Immunol. 148*, 3920−3928.
(43) Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002) Capacitative Ca^{2+} influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase. *J. Leukocyte Biol. 71*, 611−617.
(44) Gorzalczany, Y., Sigal, N., Itan, M., Lotan, O., and Pick, E. (2000) Targeting of Rac1 to the phagocyte membrane is sufficient for the induction of NADPH oxidase assembly. *J. Biol. Chem. 275*, 40073−40081.
(45) Ahmed, S., Prigmore, E., Govind, S., Veryard, C., Kozma, R., Wientjes, F. B., Segal, A. W., and Lim, L. (1998) Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox). *J. Biol. Chem. 273*, 15693−15701.
(46) Schultheis, M., Diestel, S., and Schmitz, B. (2007) The Role of Cytoplasmic Serine Residues of the Cell Adhesion Molecule L1 in Neurite Outgrowth, Endocytosis, and Cell Migration. *Cell. Mol. Neurobiol. 27*, 11−31.
(47) Chemnitz, J. M., Lanfranco, A. R., Braunstein, I., and Riley, J. L. (2006) B and T Lymphocyte Attenuator-Mediated Signal Transduction Provides a Potent Inhibitory Signal to Primary Human CD4 T Cells That Can Be Initiated by Multiple Phosphotyrosine Motifs. *J. Immunol. 176*, 6603−6614.
(48) Gavrieli, M., Watanabe, N., Loftin, S. K., Murphy, T. L., and Murphy, K. M. (2003) Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of B and T lymphocyte attenuator required for association with protein tyrosine phosphatases SHP-1 and SHP-2. *Biochem. Biophys. Res. Commun. 312*, 1236−1243.
(49) Florey, O., Durgan, J., and Muller, W. (2010) Phosphorylation of Leukocyte PECAM and Its Association with Detergent-Resistant Membranes Regulate Transendothelial Migration. *J. Immunol. 185*, 1878−1886.
(50) Tseng, S.-Y., Liu, M., and Dustin, M. L. (2005) CD80 Cytoplasmic Domain Controls Localization of CD28, CTLA-4, and Protein Kinase Cθ in the Immunological Synapse. *J. Immunol. 175*, 7829−7836.
(51) Gibson, A. W., Li, X., Wu, J., Baskin, J. G., Raman, C., Edberg, J. C., and Kimberly, R. P. (2012) Serine phosphorylation of FcγRI cytoplasmic domain directs lipid raft localization and interaction with protein 4.1G. *J. Leukocyte Biol. 91*, 97−103.
(52) Xu, W., Shy, M., Kamholz, J., Elferink, L., Xu, G., Lilien, J., and Balsamo, J. (2001) Mutations in the cytoplasmic domain of P0 reveal a role for PKC-mediated phosphorylation in adhesion and myelination. *J. Cell Biol. 155*, 439−446.
(53) Garnacho, C., Shuvaev, V., Thomas, A., McKenna, L., Sun, J., Koval, M., Albelda, S., Muzykantov, V., and Muro, S. (2008) RhoA activation and actin reorganization involved in endothelial CAM-mediated endocytosis of anti-PECAM carriers: Critical role for tyrosine 686 in the cytoplasmic tail of PECAM-1. *Blood 111*, 3024−3033.
(54) Newman, P. J., and Newman, D. K. (2003) Signal Transduction Pathways Mediated by PECAM-1. *Arterioscler., Thromb., Vasc. Biol. 23*, 953−964.
(55) Sardjono, C. T., Harbour, S. N., Yip, J. C., Paddock, C., Tridandapani, S., Newman, P. J., and Jackson, D. E. (2006) Palmitoylation at Cys595 is essential for PECAM-1 localisation into membrane microdomains and for efficient PECAM-1-mediated cytoprotection. *Thromb. Haemostasis 96*, 756−766.
(56) Paddock, C., Lytle, B. L., Peterson, F. C., Holyst, T., Newman, P. J., Volkman, B. F., and Newman, D. K. (2011) Residues within a lipid-associated segment of the PECAM-1 cytoplasmic domain are susceptible to inducible, sequential phosphorylation. *Blood 117*, 6012−6023.
(57) Lyck, R., Reiss, Y., Gerwin, N., Greenwood, J., Adamson, P., and Engelhardt, B. (2003) T-cell interaction with ICAM-1/ICAM-2 double-deficient brain endothelium in vitro: The cytoplasmic tail of endothelial ICAM-1 is necessary for transendothelial migration of T cells. *Blood 102*, 3675−3683.
(58) Greenwood, J., Amos, C. L., Walters, C. E., Couraud, P.-O., Lyck, R., Engelhardt, B., and Adamson, P. (2003) Intracellular Domain of Brain Endothelial Intercellular Adhesion Molecule-1 Is Essential for T Lymphocyte-Mediated Signaling and Migration. *J. Immunol. 171*, 2099−2108.
(59) van Buul, J. D., Allingham, M. J., Samson, T., Meller, J., Boulter, E., Garcia-Mata, R., and Burridge, K. (2007) RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration. *J. Cell Biol. 178*, 1279−1293.
(60) Pazdrak, K., Young, T. W., Stafford, S., Olszewska-Pazdrak, B., Straub, C., Starosta, V., Brasier, A., and Kurosky, A. (2008) Cross-Talk between ICAM-1 and GM-CSF Receptor Signaling Modulates Eosinophil Survival and Activation. *J. Immunol. 180*, 4182−4190.
(61) Oh, H. M., Lee, S., Na, B. R., Wee, H., Kim, S. H., Choi, S. C., Lee, K. M., and Jun, C. D. (2007) RKIKK motif in the intracellular domain is critical for spatial and dynamic organization of ICAM-1: Functional implication for the leukocyte adhesion and transmigration. *Mol. Biol. Cell 18*, 2322−2335.