Filamin B Mediates ICAM-1-driven Leukocyte Transendothelial Migration*

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During inflammation, the endothelium mediates rolling and firm adhesion of activated leukocytes. Integrin-mediated adhesion to endothelial ligands of the Ig-superfamily induces intracellular signaling in endothelial cells, which promotes leukocyte transendothelial migration. We identified the actin cross-linking molecule filamin B as a novel binding partner for intracellular adhesion molecule-1 (ICAM-1). Immune precipitation as well as laser scanning confocal microscopy confirmed the specific interaction and co-localization of endogenous filamin B with ICAM-1. Importantly, clustering of ICAM-1 promotes the ICAM-1-filamin B interaction. To investigate the functional consequences of filamin B binding to ICAM-1, we used small interfering RNA to reduce filamin B expression in ICAM-1-GFP expressing HeLa cells. We found that filamin B is required for the lateral mobility of ICAM-1 and for ICAM-1-induced transmigration of leukocytes. Reducing filamin B expression in primary human endothelial cells resulted in reduced recruitment of ICAM-1 to endothelial docking structures, reduced firm adhesion of the leukocytes to the endothelium, and inhibition of transendothelial migration. In conclusion, this study identifies filamin B as a molecular linker that mediates ICAM-1-driven transendothelial migration.

Endothelial cells are highly specialized to create a relatively impermeable barrier between the circulating blood and underlying tissue. The endothelium plays an important role in the migration of leukocytes across the vascular barrier, which is essential for host defense and is an important aspect of inflammatory diseases such as rheumatoid arthritis or atherosclerosis (1). Transendothelial migration involves initial tethering of activated leukocytes to the inflamed vessel wall, followed by the rolling of leukocytes over the endothelium (2, 3). The leukocytes then firmly adhere, which is followed by spreading and migration across the endothelium. Firm adhesion between leukocytes and the endothelium is mediated by interactions between leukocyte integrins and endothelial immunoglobulin-like adhesion molecules (Ig-CAMs), such as the intercellular cell adhesion molecule-1 (ICAM-1) (4–8).

The resting endothelium expresses ICAM-1 at low levels. However, upon activation by inflammatory stimuli the expression of ICAM-1 is markedly increased, to promote integrin-mediated adhesion and transmigration of leukocytes (9). ICAM-1 is composed of five extracellular Ig-like repeats, a single transmembrane spanning part and a short intracellular domain consisting of 28 amino acids. The cytoplasmic domain of ICAM-1 is implicated in the activation of signal transduction pathways and transendothelial migration (4, 10, 11). Clustering of ICAM-1 initiates endothelial signaling, which includes activation of small GTPases such as RhoA, Rac1, and RhoG (4, 10, 12–16) and Src-kinase (17, 18) and p38 MAP kinase (19). It is believed that these pathways contribute to the transient reduction of endothelial integrity and thereby facilitate efficient leukocyte transendothelial migration (TEM). However, the proximal signaling events induced by ICAM-1 are unknown. The short intracellular domain of ICAM-1 contains no identified signaling motifs but several proteins have been reported to interact or co-localize with ICAM-1, some of which have been implicated in actin cross-linking, such as α-actinin, ezrin, and moesin (20–23).

The interaction of leukocytes with the endothelium induces the formation of large membrane protrusions known as endothelial docking structures or transmigratory cups (6, 24). Integrin ligands such as ICAM-1 and VCAM-1 are recruited to these structures together with specific cytoskeletal and signaling molecules (6, 11). The current hypothesis is that the assembly of these structures supports the formation of multimolecule...
lar complexes facilitating endothelial signaling, important for the transmigration of leukocytes (6, 24).

In a search for proteins that mediate IgCAM signaling in endothelial cells, we used biotinylated peptides encoding the intracellular domains of ICAM-1, followed by isolation and identification of bound proteins. Here, we show that ICAM-1 clustering induced the association between ICAM-1 and filamin, a protein implicated in actin cross-linking (6, 25–28). Reducing filamin expression in primary human endothelial cells results in impaired ICAM-1 clustering and membrane dynamics and reduced adhesion and transendothelial migration of leukocytes. Our study uncovers filamin as an important component of the proximal signaling events downstream from ICAM-1 in endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293, COS-7, HL60, and HeLa were maintained in (Iscove’s modified Dulbecco’s medium) (BioWhittaker, Verviers Belgium) containing 10% heat-inactivated fetal calf serum (Invitrogen, Breda The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin at 37 °C and 5% CO2. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Baltimore, MD) and cultured during regular passaging and for experiments following fibronectin (10 μg/ml) coating of the (Sigma) tissue culture flasks (Invitrogen) or glass slides in EGM2 containing singlequots (Lonza, Baltimore, MD). Endothelial cells were cultured until passage 9. For siRNA transfection experiments early passages (<5) were used.

siRNA Transfections—for siRNA-mediated down-regulation of Caveolin-1, filamin A, and filamin B the following sequences were used: Caveolin-1, 5’-AAUCUCAUACG-GAAGCUC-3’; filamin A, 5’-CACAGAAUUUGACCAA-GAUAAGAUU-3’; filamin B, 5’-GCCCAUUACCGUGAAGAU-3’. Oligos were purchased from Eurogentec (Liege, Belgium); control siRNA was used from Dharmacon (Perbio, Etten-Leur, The Netherlands). An expression construct for ICAM-1-GFP was kindly provided by Dr. F. Sanchez (University of Madrid, Madrid, Spain), hemagglutinin-tagged filamin B and filamin A repeats 19–24 as well as the GST-filamin B-(19–24) and the GST-filamin B-var-(19–24) were a kind gift from Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands). Double strand siRNA oligos were transfected in HUVEC using Interferin transfection reagent and transfection medium (Tebu-Bio, Heerhugowaard, The Netherlands). Double strand siRNA oligos were transfected in HUVEC using Interferin transfection reagent and transfection medium (Tebu-Bio, Heerhugowaard, The Netherlands) according to the manufacturer’s protocol. Oligofectamine (Invitrogen) was used to transfect siRNA oligos and expression constructs in HeLa cells. Cells were transfected when ~70% confluent, 0.300 μmol of siRNA was diluted in 250 μl of Opti-MEM and left for 5 minutes at room temperature. 15 μl of Oligofectamine was mixed with 60 μl of Opti-MEM and incubated for 5 minutes at room temperature. Next, the two mixtures were pooled and incubated for 20 minutes at room temperature. The mixture was then added to the cells for 6 hours and replaced with fresh culture medium. Both HeLa cells and HUVEC were used for experiments 48 hours after transfection.

Antibodies—The monoclonal antibody (mAb) to ICAM-1 and anti-ICAM-1-fluorescein isothiocyanate were purchased from R&D Systems (Minneapolis, MN); the anti-VCAM-1 mAb was from Beckman Coulter (Marseille, France); the goat polyclonal Ab to VCAM-1 (C-19) was purchased from Tebu-Bio. Filamin A mAb was purchased from Serotec (Oxford UK); polyclonal filamin B Ab was from Millipore (Amsterdam, The Netherlands); the polyclonal caveolin-1 Ab was from BD Transduction Laboratories; mAb anti-hemagglutinin (12CA5) was from Boehringer (Almere, The Netherlands); isotype control IgG was from Sanquin (Amsterdam, The Netherlands); Alexa 488-labeled chicken anti-mouse, Alexa 488 chicken antirabbit, Alexa 594 chicken anti-mouse, Alexa 594 chicken anti-rabbit, and Alexa 647 chicken anti-goat were purchased from Invitrogen. For F-actin staining BODIPY-650/665-phalloidin and Texas Red-phalloidin were used (Invitrogen).

Coating of Beads—Magnetic goat anti-mouse IgG-coated Dynabeads (Invitrogen) were coated with ICAM-1 mAb, VCAM-1 mAb, or IgG isotype control according to the manufacturer’s protocol. In short, beads were washed with ice-cold PBS (+0.1% BSA, 2 mM EDTA) and incubated with 1 μg of antibody per 1 × 107 beads for 45 minutes at 4°C under constant head over head rotation. The beads were subsequently washed twice to remove any unbound antibody. Polystyrene 10 and 3-μm beads (Polysciences Inc., Warrington, PA) were coated with antibody according to the manufacturer’s instructions. Beads were washed in PBS and incubated overnight with glutaraldehyde at room temperature under constant rotation. Beads were then washed three times with PBS and incubated with 1 μg of antibody per 2 × 106 beads for 4–5 hours. After washing, the beads were incubated in 0.5 M ethanolamine in PBS for 30 minutes and subsequently washed and blocked with 10 mg/ml BSA in PBS for 30 minutes.

SDS-PAGE, Western Blotting, and Silver Staining—SDS-PAGE samples were analyzed on 7.5, 10, or 12.5% polyacrylamide gels depending on the size of the proteins of interest and transferred onto PVDF membrane (Bio-Rad). Following blocking in 5% low fat milk in TBST (Tris-buffered saline, Tween 20) the blots were incubated with the primary antibody overnight at 4°C, washed 3 times for 10 minutes in TBST, and subsequently incubated with horseradish peroxidase-coupled secondary antibodies (dilution: 1:7000) in TBST for 1.5 hours at room temperature followed by washing 3 times with TBST for 20 minutes each and development of the blot by ECL (GE Healthcare). For silver staining, and subsequent mass spectrometry, pre-cast gradient 4–12% Bis-Tris gel (Invitrogen) was used according to manufacturer’s instructions.

In-gel Digestion and Mass Spectrometry—Protein bands were excised from SDS-PAGE gels, reduced, alkylated, and in-gel digested using trypsin (modified, sequencing grade, Promega) as previously described (40). After digestion, peptides were collected using two rounds of extraction with 20 μl of 0.1% trifluoroacetic acid and stored at ~20°C prior to analysis by mass spectrometry. For LC-MS analysis, samples were injected onto a nano-LC system (Ultimate, Dionex, Amsterdam, The Netherlands) equipped with a peptide trap column (Pepmap 100, 0.3 inner diameter × 1 mm) and an analytical column (Pepmap 100, 0.075 inner diameter × 150 mm, Dionex). The mobile phases consisted of (A) 0.04% formic acid, 0.4% acetonitrile and (B) 0.04% formic acid, 90% acetonitrile. A 45-min linear gradient from 0 to 60% B was applied at a flow rate of 0.2 μl/min. The
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outlet of the LC system was coupled to an HCT ion-trap mass spectrometer (Bruker Daltonics, Bremen) using a nano-electrospray ionization source. Eluting peptides were analyzed in the data dependent MS/MS mode over a 400–1600 m/z range. Mass spectra were evaluated using the Data Analysis 3.1 software package (Bruker Daltonics, Bremen, Germany). MS/MS spectra were searched against the human IPI data base using the Mascot search algorithm (Matrix Science, London, UK), allowing mass tolerances of 1.5 Da for MS and 0.5 Da for MS/MS and one missed cleavage site. Carbamidomethylcysteine was taken as a fixed modification and oxidation of methionine as a variable modification.

Pull-down Assay and Immunoprecipitation—A synthetic, biotinylated peptide encoding the intracellular domain of human ICAM-1, VCAM-1, or empty beads were used in pull-down assays as previously described (41). The following sequences were synthesized from the N to C terminus: ICAM-1, NH2–RQRKIKKYRLQQAQKGTPMKPNTQATPP–COOH; VCAM-1, NH2–IIFYARKMKGSYSLVEAQSKV–COOH. A confluent HUVEC monolayer in a 100-mm Petri dish was washed with cold PBS (1 mM CaCl2, 0.5 mM MgCl2) and lysed in cold Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl2, 10% glycerol, 10% DMSO, and 1% Nonidet P-40 (v/v)) supplemented with protease inhibitors (complete mini EDTA, Roche) for 5 min. After lysis, cell debris was removed by centrifugation (14,000 × g, 5 min at 4 °C). Supernatant was incubated with 5 μg of biotinylated peptide comprising the intracellular tail of ICAM-1 or indicated controls and 30 μl (concentration of 2.4 mg/ml packed gel) of streptavidin beads for 2–3 h under constant head over head rotation at 4 °C. Beads were subsequently washed five times with Nonidet P-40 lysis buffer and resuspended in SDS-PAGE sample buffer. Alternatively, antibody-coated magnetic beads were resuspended in EGM2 and incubated on a HUVEC monolayer at a concentration of ~4–8 beads per cell for 30 min unless otherwise indicated. For subsequent immunoprecipitation, cells were gently washed in cold PBS (supplemented with 1 mM CaCl2, 0.5 mM MgCl2) to remove unbound beads and lysed in cold RIPA buffer (1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 50 mM Tris, pH 7.4, 1% deoxycholate, and 0.1% SDS). Beads were extracted from cell lysates using a magnetic pen (PickPen 1M, BioNobile), and subsequently washed five times in a Nonidet P-40-based lysis buffer (1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 50 mM Tris, pH 7.4) to reduce coagulation of the beads, 15 units of DNase I (Fermentas, Germany) was added to the lysis buffer and incubated at 37 °C for 5 min and transferred onto ice again. Next, the cells were washed twice with ice-cold PBS and subsequently fixed with 4% formaldehyde in PBS. Confocal microscopy was used to make overviews at fixed positions in the coverslips. Subsequently the total number of beads were counted using Zeiss LSM Image software.

Confocal Laser Scanning Microscopy—For immunofluorescence, HUVECs or HeLa ICAM-1-GFP cells were grown on fibronectin-coated 14- or 30-mm coverslips. HeLa-expressing ICAM-1-GFP cells, transfected with control or filamin B siRNA were cultured on the coverslips to confluence. Cells were left on ice for 3 min and equal amounts of anti-ICAM-1-coated beads (10 μm diameter) were added. Cells were left on ice for an additional 3 min to allow sedimentation of the beads onto the monolayer. Subsequently the beads were incubated at 37 °C for 5 min and transferred onto ice again. Next, the cells were washed twice with ice-cold PBS and subsequently fixed with 4% formaldehyde in PBS. Confocal microscopy was used to make overviews at fixed positions in the coverslips. Subsequently the total number of beads were counted using Zeiss LSM Image software.

Transmigration and Adhesion Assay—Transmigration was analyzed using Transwell permeable supports from Costar (d = 6.5 mm; 5 μm pore size). A monolayer of HeLa ICAM-1-GFP expressing cells were cultured on the filter and transfected with siRNA for filamin B or with control siRNA (Dharmacon). The cells were seeded at 2 × 105 cells per well 24 h after transfection and subsequently cultured for an additional 24 h. The monolayer was then washed once with migration medium (Iscove’s modified Dulbecco’s medium) 2% FCS without antibiotics and 1 × 105 differentiated HL60 cells (4 days, 1.3% DMSO supplemented to culture medium) were transferred to the upper compartment and cells were allowed to migrate for 2 h toward the lower chamber in a chemotactic gradient with SDF-1. The number of migrated HL60 cells is quantified by flow cytometry using count beads according to van Hennik et al. (42). Following the migration assay, the monolayer was fixed, permeabilized, stained with Texas Red-phalloidin to visualize F-actin and analyzed by microscopy to ensure that the monolayer was confluent.

Transmigration under Flow—Primary HUVECs were cultured to confluence in EGM2 (Cambrex) on fibronectin-coated glass coverslips transfected with either control or filamin B siRNA according to manufacturer’s protocol using siRNA transfection reagent (Santa Cruz, Santa Cruz, CA). Cells were treated with 10 ng/ml TNF-α overnight. Next, cells were mounted onto the microscope stage using a POC-mini chamber system (LaCon, Staig, Germany) and connected to a perfusion pump. Using physiological flow conditions (5 dyn/cm2), 1 × 106 differentiated HL60 cells/ml were perfused over the endothelial cells, followed by another 20 min of fluid-flow. Transmigration was characterized by adhesive cells crossing the monolayer during this time frame, changing from a bright to a dim appearance (see Fig. 8C). Migrated cells were quantified by counting per field-of-view. From one experiment, five fields were analyzed.

Adhesion of anti-ICAM-1 antibody-coated beads was analyzed in a 24-well plate containing fibronectin-coated glass coverslips. HeLa-expressing ICAM-1-GFP cells, transfected with control or filamin B siRNA were cultured on the coverslips to confluence. Cells were left on ice for 3 min and equal amounts of anti-ICAM-1-coated beads (10 μm diameter) were added. Cells were left on ice for an additional 3 min to allow sedimentation of the beads onto the monolayer. Subsequently the beads were incubated at 37 °C for 5 min and transferred onto ice again. Next, the cells were washed twice with ice-cold PBS and subsequently fixed with 4% formaldehyde in PBS. Confocal microscopy was used to make overviews at fixed positions in the coverslips. Subsequently the total number of beads were counted using Zeiss LSM Image software.
trols were seeded on 30-mm coverslips and transfected with filamin B siRNA and siGlow (Dharmacon) according to the manufacturer’s instructions. Coverslips were transferred to the POC-mini chamber system and mounted onto a heating block connected to a confocal microscope (Zeiss LSM510). FRAP (fluorescent recovery after photobleaching) experiments were performed using 30 iterations with 488-nm laser illumination, at maximum power (25 milliwatts). Fluorescence recovery was measured by time lapse imaging. Image analysis was performed with LSM 510 software (Carl Zeiss MicroImaging, Inc.). GraphPad Prism 4 was used for statistical analysis and nonlinear regression. For curve fitting a one exponential association was used (equation; $Y = Y_{\text{max}} \left(1 - \exp(-K \times X)\right)$), which starts at zero and ascend to $Y_{\text{max}}$ with a rate constant $K$ in which $Y_{\text{max}}$ represents the mobile fraction and $K$ represents the time characteristics of the curve.

RESULTS

Identification of Proteins Associating to the Intracellular Domain of ICAM-1—Although the intracellular domain of ICAM-1 is short (28 amino acids) and contains no identified signaling domains, it has been shown that the lack of the intracellular tail of ICAM-1 results in a decrease of leukocyte transmigration and a loss of formation of apical cups around adhered leukocytes (4, 10, 16, 29). These results indicate that the ICAM-1 intracellular tail initiates signaling pathways involved in TEM. To identify proteins that bind to the intracellular domain of ICAM-1 we performed pull-down assays with lysates of primary HUVEC using biotinylated peptides encoding the intracellular tail of ICAM-1. Following capture with streptavidin-coated beads, peptide-bound proteins were separated by SDS-PAGE and visualized by silver staining (Fig. 1A). A peptide encoding the VCAM-1 intracellular domain was used as a specificity control. The most prominent protein band specifically interacting with the ICAM-1 peptide (migrating at an molecular mass above 200 kDa) was analyzed by mass spectrometry and identified as the cytoskeletal linker protein filamin B.

To confirm the interaction of filamin B with the intracellular domain of ICAM-1, peptide-binding proteins were separated by SDS-PAGE and filamin B was identified by Western blot. Fig. 1B shows that filamin B specifically binds to the intracellular domain of ICAM-1 and not to the intracellular domain of VCAM-1 or to streptavidin beads. There are three filamin homologues expressed in human, filamin A and filamin B, which are ubiquitously expressed, and a muscle-specific filamin C. Because filamin A and B share 70% sequence homology, we also tested the binding of filamin A to the ICAM-1 cytoplasmic domain. Like filamin B, filamin A also interacts with the cytoplasmic domain of ICAM-1, albeit less efficiently (Fig. 1B). In addition to filamin B, we detected another protein of ~20 kDa binding to ICAM-1 (Fig. 1A). This protein was identified by Western blotting as caveolin-1. Recently, caveolin-1 has been reported to play a key role in ICAM-1-mediated transendothelial migration of T-lymphocytes (30). Moreover, caveolin-1 was previously reported to interact with filamin A and filamin B (30, 31). Thus, ICAM-1 may form a complex with filamin proteins and caveolin-1 to mediate leukocyte transmigration.

Filamin is a 280-kDa protein comprising an N-terminal actin-binding domain and a rod domain composed of 24 homologous repeats. We tested which region in filamin is involved in ICAM-1 binding. Because most reported interactions involve the C-terminal region of filamin B, we transfected a construct encoding repeats 19–24 of filamin B in COS-7 cells and performed a pull-down experiment with the C-terminal peptide of ICAM-1. The results in Fig. 1C showed that the filamin B (19–24) construct binds the ICAM-1 peptide (Fig. 1C). A construct containing domains 19–24 of filamin A also interacts with the ICAM-1 peptide (data not shown). To address if this region of filamin B binds directly to the intracellular tail of ICAM-1, a GST fusion protein encoding filamin B domains 19–24 was isolated and incubated with C-terminal peptides of either ICAM-1 or VCAM-1. Using streptavidin beads, peptide-binding proteins were precipitated and blotted for GST. The results showed that the ICAM-1 C-terminal region directly interacts with filamin B (Fig. 1D). The VCAM-1 peptide showed only low binding to the GST-filamin B (19–24) domain. Interestingly, the peptides did not interact with a splice variant of filamin B, lacking amino acids 2082–2122 (GST-filamin B-var-1-(19–24) were isolated and incubated with the ICAM-1 or VCAM-1 C-terminal peptides; bound proteins were analyzed by Western blot.
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Endogenous Filamin and Caveolin-1 Interact with Endogenous ICAM-1—To study the interaction between endogenous ICAM-1 and filamin B, TNF-α-stimulated primary HUVEC were used. Western blot and flow cytometry analyses confirmed that overnight stimulation with TNF-α induced an increase in ICAM-1 expression in HUVEC (Fig. 2A). Primary HUVEC were grown to confluence on fibronectin-coated dishes, stimulated with TNF-α overnight, and incubated for 30 min with magnetic αICAM-1 antibody-coated beads to induce clustering of ICAM-1. The cells were lysed and the magnetic beads were extracted from the lysate using a magnetic particle concentrator pen. SDS-PAGE followed by Western blot analysis of bead-associated proteins demonstrated that endogenous ICAM-1 could be effectively isolated using this method (Fig. 2B). Likewise, αVCAM-1 antibody-coated beads but not IgG could extract VCAM-1 protein (Fig. 2B). A small amount of ICAM-1 was repeatedly found associated to VCAM-1.

Analysis of the binding of filamin B and caveolin-1 demonstrated the co-precipitation of these proteins with the αICAM-1-coated beads, whereas these proteins were not detected using αVCAM-1, αIgG isotype control, or α major histocompatibility complex antibody-coated beads (Fig. 2C and data not shown). Interestingly, addition of the αICAM-1 antibody-coated beads to an endothelial cell lysate did not result in the co-precipitation of detectable amounts of filamin B (Fig. 2D), indicating that the interaction with endogenous filamin B is promoted upon clustering of ICAM-1.

Caveolin-1 Binds Indirectly to ICAM-1—To determine whether filamin is required for the caveolin-1-ICAM-1 interaction, a pull-down assay using the ICAM-1 C-terminal peptide was performed with lysates of cells treated with siRNAs for filamin A, B, or caveolin-1. In cells with reduced caveolin-1 levels, the interaction of filamin A and B with the ICAM-1 cytoplasmic tail remained intact (Fig. 3). Knockdown of filamin A reduced the binding of caveolin-1 to the ICAM-1 peptide, whereas reducing filamin B had little to no effect on the binding of caveolin-1 to the ICAM-1 C terminus (Fig. 3). These data suggest that the ICAM-1-caveolin-1 interaction is primarily mediated through filamin A. Although it has been reported that filamin B also binds caveolin-1 (30, 31), we could not detect a significant contribution of filamin B to the ICAM1-caveolin-1 interaction.

Filamin Co-localizes with ICAM-1 Upon ICAM-1 Clustering—Because filamin B was initially identified as the major isoform that bound the ICAM-1 C terminus (Fig. 1A), we focused in subsequent experiments on filamin B. The distribution of ICAM-1 and filamin B was analyzed by immunocytochemistry in combination with confocal microscopy. Filamin B staining showed minimal co-localization with ICAM-1 (Fig. 4A). However, upon cross-linking of ICAM-1 using soluble αICAM-1 antibodies, ICAM-1 is redistributed to cell borders and to punctated structures on the cell body, in good agreement with findings by others (33). Leukocyte adhesion to the endothelial surface results in the recruitment of ICAM-1 to sites of adhesion (16, 23, 24, 29, 31). To mimic leukocyte binding via ICAM-1, we incubated primary HUVEC with αICAM-1 antibody-coated beads and found that filamin B was recruited to these beads, co-localizing with ICAM-1 at sites of adhesion.

FIGURE 2. Filamin B interacts with endogenous ICAM-1. A, ICAM-1 protein is up-regulated after TNF-α treatment in primary human endothelial cells. Left panel shows Western blot analysis of ICAM-1 expression in HUVEC. TNF-α treatment is in hours. Actin is included as a loading control. The bar graph shows the expression of ICAM-1, analyzed by flow cytometry. MFI, mean fluorescence intensity. Data are representative for at least three independent experiments. B, magnetic beads coated with anti-ICAM-1 or -VCAM-1 antibodies or with control IgG were incubated for 30 min on a monolayer of TNF-α-stimulated HUVEC, followed by lysis. The beads were used for specific immunoprecipitation (IP) of the various indicated proteins. C, proteins were isolated from cell extracts as described under B and IPs were analyzed for the presence of filamin B or caveolin-1. Filamin B levels in the lysates are included as loading controls. D, anti-ICAM-1 antibody-coated magnetic beads were added to intact cells (pre-lysis), or to endothelial cell lysates (post-lysis). ICAM-1 associates with filamin B when clustered by anti-ICAM-1 antibody-coated magnetic beads (left lane). ICAM-1 antibody-coated magnetic beads added post-lysis bound ICAM-1, but did not efficiently co-precipitate filamin B (left lanes). Lower panel shows the loading control for filamin B. Data are representative for three independent experiments.
Similar results were obtained when differentiated HL-60 cells were used (supplemental Fig. S1). To control for the specificity of the staining, we used H9251 VCAM-1 or H9251 major histocompatibility complex antibody-coated beads that also adhered to activated endothelial cells. The results showed that these beads recruited filamin B only to a limited extent (Fig. 4B and data not shown), which is in agreement with the biochemical data in Fig. 2D. This indicates that efficient filamin B recruitment depends on ICAM-1 clustering.

**ICAM-1-GFP Expression in HeLa Cells**—To study the functional relevance of the interaction of filamin B with ICAM-1, we used HeLa cells that stably expressed ICAM-1-GFP. HeLa cells express only very low levels of endogenous ICAM-1, compared with TNF-α-stimulated endothelial cells (data not shown). Western blot analyses showed that the transfected cells express the ICAM-1-GFP protein (Fig. 5A) and flow cytometry analysis indicated that ~90% of HeLa cells stably expressed ICAM-1-GFP (dark line). Gray line shows background staining of IgG-isotype control. C, HeLa ICAM-1-GFP expressing cells (left panel) recruit ICAM-1-GFP to sites of ICAM-1 antibody-coated beads (asterisks). The right panel shows the location of the beads by differential interference contrast (DIC). Bar, 10 μm. D, Z-stack analysis of ICAM-GFP, recruited around an adherent antibody-coated bead. Projection shows a tilted image of the cup structure, formed around the bead.
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siRNA efficiently reduced the levels of endogenous filamin B in these cells (Fig. 6A). We initially analyzed the role of filamin B in ICAM-1 membrane dynamics. To this end we used FRAP analysis. FRAP analysis of the membrane-associated GFP-CAAX protein revealed no difference in the fluorescence recovery between filamin B-siRNA-treated cells and control cells (Fig. 6A). FRAP analysis of ICAM-1-GFP showed that reduced expression of filamin B resulted in a decreased fluorescence recovery of ICAM-1-GFP (Fig. 6B, see also supplementary Videos 2 and 3). This data suggest that filamin B regulates ICAM-1-GFP mobility in the plasma membrane.

Note from the videos that the distribution of ICAM-1-GFP in the filamin B siRNA-treated cells is much more diffuse throughout the plasma membrane, when compared with the controls, which show more discrete apical structures in which ICAM-1-GFP is concentrated.

Because filamin B is an adaptor protein that is linked to the subcortical actin cytoskeleton (34), filamin may regulate cytoskeletal dynamics and thereby directly or indirectly control the mobility of ICAM-1. Detailed analysis of the data showed that reduction of filamin B expression significantly reduced the mobile fraction of ICAM-1 but not of the GFP-CAAX (Fig. 6C). Interestingly, by calculating the slope of the curves, depicted in Fig. 6, A and B, the data indicated that filamin B knockdown did not affect the speed of recovery (Fig. 6D). Finally, we did not observe major changes in cytoskeletal architecture following treatment of the endothelial cells with filamin B siRNA. Together, these data suggest that filamin B links ICAM-1 to the actin cytoskeleton and that filamin B promotes the extent rather than the speed of ICAM-1 lateral mobility within the plasma membrane, without directly affecting cytoskeletal dynamics.

Filamin Mediates Efficient Recruitment of ICAM-1 Upon Clustering—Next, we tested whether filamin B is required for ICAM-1 clustering. Recruitment of ICAM-1 to αICAM-1 antibody-coated beads was analyzed by time lapse confocal laser scanning microscopy using HeLa cells stably expressing ICAM-1-GFP in the absence or presence of filamin B. For the immunofluorescence experiments, siGLO was transfected together with filamin B siRNA, to certify that the cells used for imaging were indeed transfected with siRNA (data not shown). The antibody-coated beads induced recruitment of ICAM-1-GFP in control siRNA-treated cells within ~8 min (Fig. 7A see also supplementary Video 4). GFP-CAAX, used as a control, was not significantly recruited to ICAM-1 antibody-coated beads (data not shown). Reducing filamin B expression with siRNA revealed that ICAM-1 was eventually recruited to adherent beads, albeit with a delay of 10–15 min (see also supplementary Video 5). To complement the analysis in Fig. 7A, the contribution of filamin for the formation of cup structures was quantified using cells treated with control or filamin B siRNA. In the absence of filamin B, a significant reduction in the number of cup structures formed was found (Fig. 7B). Together, these data indicate that filamin B controls lateral motility of ICAM-1 and thereby mediates its recruitment to sites of ICAM-1 engagement.

Filamin Is Required for ICAM-1-mediated Transendothelial Migration—To test if filamin plays a role in
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ICAM-1-mediated transmigration of leukocytes, HeLa cells expressing ICAM-1-GFP (Fig. 8B) was decreased in filamin B-deficient cells. Next, differentiated HL60 cells were allowed to migrate across ICAM1-GFP expressing HeLa cells toward the chemokine SDF-1 in a Transwell-based chemotaxis assay. The results showed that introducing ICAM-1-GFP into HeLa cells was sufficient to stimulate leukocyte chemotaxis toward SDF-1 (Fig. 8B). Reducing filamin B protein levels resulted in an inhibition of ICAM-1-induced migration of leukocytes, close to levels of SDF-1-induced migration across HeLa cells that did not express ICAM1-GFP (Fig. 8B). To study the role of filamin B on the function of endogenous ICAM-1, filamin B expression was reduced in primary endothelial cells using siRNA. Subsequently, transmigration of cells across the endothelium was recorded under flow, and quantified based on their transition from phase-bright to phase-dim (Fig. 8C). These experiments showed that transendothelial migration of leukocytes in the absence of filamin B was reduced (Fig. 8D). Note that also the initial adhesion of the leukocytes to ICAM-1 was affected in filamin B-deficient primary endothelial cells (Fig. 8D). These data show that filamin B plays an important, non-redundant role in the adhesion and subsequent transmigration of leukocytes across the endothelium by regulating ICAM-1 recruitment and function.

DISCUSSION

TEM of leukocytes is the result of a dynamic interplay between activated leukocytes and endothelial cells. Leukocytes bind through integrins to their ligands, such as ICAM-1, expressed on endothelial cells, which is crucial for efficient TEM of various types of leukocyte (8) (12, 35). The binding to ICAM-1 results in ICAM-1 clustering and subsequent induction of intracellular signaling, facilitating the passage of leukocytes (11). ICAM-1 activates a wide range of signaling pathways, including protein tyrosine phosphorylation, Rho-like GTPase activation, and modulation of cytoskeletal dynamics. Most of these signaling events apparently also feed back to ICAM-1, as these have all been implicated in ICAM-1 clustering, leukocyte adhesion, and TEM.

The ICAM-1-Filamin Interaction—The ICAM-1 intracellular domain is critical for ICAM-1 function and signaling and deleting this region impairs transmigration of leukocytes (4, 10, 12, 35). However, the fact that the ICAM-1 intracellular domain is very small, lacking any established signaling motifs, raises questions about the nature of protein-protein interactions that govern ICAM-1-driven signaling. In this study we show that the intracellular domain of ICAM-1 binds the actin cross-linking proteins filamin A and, in particular, filamin B. This interaction is specific, in that the intracellular domain of VCAM-1 does not bind to filamin B. Moreover, filamin B binding to ICAM-1 is stimulated by antibody-mediated clustering, suggesting that the ICAM-1-filamin B association is induced following the binding of leukocytes to endothelial cells. The ICAM-1-filamin complex also comprises caveolin-1. Our data suggest that caveolin-1 associates to ICAM-1 through filamin, in particular filamin A. Whether indeed two functionally distinct complexes, comprising filamin B and filamin A-caveolin-1, form upon ICAM-1 clustering is as yet unknown and will be a topic of future studies.

Millán and colleagues (30) reported that ICAM-1 travels with the leukocyte to the basolateral site of the endothelial cells. Leukocytes that use the transcellular route, i.e. through the endothelial cells, use caveolae as a starting point. This pathway depends on caveolin protein, because reduced expression of caveolin results in an inhibition of transcellular migration. Our data link caveolin to ICAM-1 through filamin, in line with a recent report that showed that caveolin and filamin interact...
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In addition, the fact that filamin B binding to ICAM-1 is increased upon ICAM-1 clustering is in agreement with the data by Millán and co-workers (30), who showed that the F-actin rings, formed around adhered leukocytes, are most likely mediated by filamin. Finally, our findings are also similar to what was described for the CD4-filamin A interaction, which is increased upon CD4 ligation (36).

Recently, it was published that the intracellular tail of ICAM-5 binds to α-actinin as well as to filamin (37). However, by mapping the binding sites, it appears that α-actinin binds to the positively charged amino acid region in ICAM-5, similar as is shown for its binding to ICAM-1 (35), but filamin did not bind this region, and may therefore bind to a more membrane-distal sequence in the ICAM-1 C terminus. As for filamin B, our results show that the repeats 19–24 of filamin B, in particular repeats 19 and 20, are involved in the binding to the intracellular tail of ICAM-1. This conclusion is based on the pull-down assays using the GST-filamin B-variant construct, which lacks amino acids 2082–2122 and appears to bind more strongly than the wild-type filamin B to different integrin β subunits (32). Our experiments show that the intracellular tail of ICAM-1 did not directly associate with the filamin B-variant construct, but did associate with the 19–24 repeats of filamin B wild type.

Thus, our data show that filamin B associates, as α-actinin and ERM proteins, with the intracellular domain of ICAM-1. Whether these membrane-proximal complexes comprising ICAM-1 form in parallel or in sequence or perhaps even at different positions in the plasma membrane following ICAM-1 clustering is presently unclear. Importantly, filamin B appears to serve a critical, non-redundant role following ICAM-1 clustering. This can be concluded from our findings that the ICAM-1 recruitment around anti-ICAM-1-coated beads, as well as the adhesion and transendothelial migration of leukocytes was reduced in primary endothelial cells expressing reduced levels of filamin B, even though these cells express filamin A.

Filamin in ICAM-1 Mobility and Signaling—Clustering of ICAM-1 results in an increase in stress fiber formation and transient activation of the small GTPase RhoA (13), indicating that ICAM-1 signals toward the actin cytoskeleton. Filamins are excellent candidates in mediating this signaling as these proteins act as actin-binding scaffolds, interacting with cell-surface receptors such as integrins as well as signaling molecules such as caveolin-1, protein kinase C, and small GTPases (26, 38). Preliminary findings showed that ICAM-1 cross-linking reduces endothelial cell-cell contact in a filamin B-dependent fashion (data not shown). The signaling function of filamin may well play a role in this pathway, as we and others have previously shown that various proteins such as small GTPases and protein kinases play a key role in the modulation of cell-cell contact through Ig-CAM-driven signaling (16). Intriguingly, our FRAP experiments showed that siRNA-mediated knockdown of filamin B reduces the mobile fraction of ICAM-1 in the membrane. This is in line with the reduced recruitment of ICAM-1 to antibody-coated beads in the absence of filamin B and suggests that filamin B-controlled cytoskeletal dynamics and/or its binding to ICAM-1, promotes ICAM-1 translocation in the plane of the membrane. Thus, ICAM-1 mobility is apparently driven from within the cell, depends on a proper interaction with the cortical actin cytoskeleton network, and is enhanced after cross-linking, which promotes filamin B binding. This notion is in agreement with data by Yang et al. (39), who showed that Src-mediated phosphorylation of the actin cross-linker cortactin regulates ICAM-1 mobility. Similar as for filamin B, reduced cortactin expression impaired clustering of ICAM-1.

Recent data indicate that F-actin/filamin networks are stiffer and less dynamic compared with F-actin/α-actinin networks (27). Our data show that clustering of ICAM-1 promotes the association of filamin to ICAM-1 and that reduced filamin levels decreased the adhesion of leukocytes to ICAM-1 (Fig. 8D). This suggests that a more rigid actin network, regulated by filamin, is required for optimal ICAM-1 function, i.e. the binding of leukocytes.

Whereas binding to actin cross-linking proteins thus promotes ICAM-1 recruitment, blocking actin polymerization by cytochalasin D also increases the mobile fraction of ICAM-1 upon clustering. Similarly, under these conditions, the formation of apical cup structures is impaired, although the adhesion of leukocytes remains unaltered (24). An emerging concept is therefore that the ICAM-1-cytoskeleton connection represents a bidirectional signaling module that coordinates the mobility of IgCAMs such as ICAM-1, which, upon clustering, signal toward actin and mediates increased actin polymerization. This may likely represent a positive feedback loop that serves not only to recruit ICAM-1, but also to secure a coordinated link to a more rigid cortical actin network, which is required for proper adhesion and efficient migration of leukocytes over the endothelial apical membrane. This molecular interplay may in fact be quite similar to the situation for integrin-mediated adhesion and consequent formation of focal adhesions, which promote as well as depend upon the interaction with the actin cytoskeleton.

The above results put filamin B forward as a crucial regulator for ICAM-1 function in the process of transendothelial migration of leukocytes. Filamin B not only binds to and regulates ICAM-1 lateral mobility, but also, through its cross-linking activity, promotes formation of a rigid actin network that supports ICAM-1-mediated leukocyte extravasation, an event that is critical in immune surveillance and inflammation.

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