Polarization and interaction of adhesion molecules P-selectin glycoprotein ligand 1 and intercellular adhesion molecule 3 with moesin and ezrin in myeloid cells


In response to the chemoattractants interleukin 8, C5a, N-formyl-methionyl-leucyl-phenylalanine, and interleukin 15, adhesion molecules P-selectin glycoprotein ligand 1 (PSGL-1), intercellular adhesion molecule 3 (ICAM-3), CD43, and CD44 are redistributed to a newly formed uropod in human neutrophils. The adhesion molecules PSGL-1 and ICAM-3 were found to colocalize with the cytoskeletal protein moesin in the uropod of stimulated neutrophils. Interaction of PSGL-1 with moesin was shown in HL-60 cell lysates by isolating a complex with glutathione S-transferase fusions of the cytoplasmic domain of PSGL-1. Bands of 78- and 81-kd were identified as moesin and ezrin by Western blot analysis. ICAM-3 and moesin also coeluted from neutrophil lysates with an anti-ICAM-3 immunoaffinity assay. Direct interaction of the cytoplasmic domains of ICAM-3 and PSGL-1 with the amino-terminal domain of recombinant moesin was demonstrated by protein-protein binding assays. These results suggest that the redistribution of PSGL-1 and its association with intracellular molecules, including the ezrin-radixin-moesin actin-binding proteins, regulate functions mediated by PSGL-1 in leukocytes stimulated by chemoattractants. (Blood. 2000;95:2413-2419)

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Introduction

Leukocytes emigrating from the bloodstream toward an inflammatory focus follow different chemoattractant signals that guide their path. An early response of these cells to such stimuli is a transition from a spherical to a polarized morphologic conformation, which occurs as soon as the cell begins to move. This response also occurs with several chemokines that induce both leukocyte activation and migration. Interleukin 8 (IL-8) belongs to the subfamily of CXC chemokines that mainly attract neutrophils. It is released in response to inflammatory stimuli and induces the transmigration of neutrophils across vascular endothelium. CXC chemokattractants induce the full range of neutrophil activation events, including oxygen free-radical production and degranulation. In contrast, other neutrophil-activating agents, such as tumor necrosis factor α (TNF-α) do not act as primary chemotactic factors and induce degranulation only after priming with other agents. Similar to the classic leukocyte chemoattractants C5a and N-formyl peptides, chemokines bind to 7-trans-membrane spanning receptors coupled to a heterotrimeric G-protein signaling pathway.

On activation, endothelial cells express specific adhesion molecules that are able to bind free-floating neutrophils. The initial contact and rolling of neutrophils along the endothelium are predominantly mediated by E- and P-selectins, which are expressed by activated endothelium, and by L-selectin, which is constitutively expressed on leukocytes. All 3 selectins bind, in a calcium-dependent manner, to sialylated and fucosylated oligosaccharides such as sialyl Lewis x and its isomer sialyl Lewis a, which induces the full range of neutrophil activation events, including oxygen free-radical production and degranulation. In contrast, other neutrophil-activating agents such as tumor necrosis factor α (TNF-α) do not act as primary chemotactic factors and induce degranulation only after priming with other agents. Similar to the classic leukocyte chemoattractants C5a and N-formyl peptides, chemokines bind to 7-trans-membrane spanning receptors coupled to a heterotrimeric G-protein signaling pathway.

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membrane ruffling.\textsuperscript{29-32} Suppression of their expression has prof
found effects on cell functions.\textsuperscript{33} Ezrin and moesin are also 
expressed in neutrophils,\textsuperscript{34} and because activation with different
chemoattractants and movement depend on adhesion and on the actin 
cytoskeleton, we explored the distribution of a variety of adhesion 
molecules and moesin and possible interactions between them. We 
found that several receptors were distributed to the uropod of migrating 
neutrophils, where they were codistributed with moesin. Moreover, 
we observed that, in vitro, the aminoterminal domain of moesin interacted 
directly with the cytoplasmic domain of at least 2 of these receptors—ICAM-3 and PSGL-1.

### Materials and methods

#### Antibodies, chemokines, and reagents

The anti-ICAM-3 HP2/19 and TP1/25, anti-ICAM-1 MEM 111, anti-CD44 
HP2/19, anti-CD43 TP1/36, anti-CD45 D3/9, anti-PSGL-1 KPL1, and 
anti-moesin/antiadixdin 38/37 monoclonal antibodies (mAbs) were 
described previously.\textsuperscript{17,25,35,36,37} Antimoesin 95/2 and the antiezrin 90/3 
polyclonal antisera (pAb) were raised in rabbits by immunization with 
recombinant human moesin and purified human ezrin, respectively.\textsuperscript{29,38} 
Antivinculin mAb was purchased from Sigma Chemical Co (St Louis, 
MO). Goat anti-HPGRF78 (HSP78) pAb was purchased from Santa Cruz 
Biotechnology, Inc (Santa Cruz, CA). Aprotinin, iodoacetamide, C5a, human 
recombinant TNF-α, and FMLP were purchased from Sigma. IL-8 and interleukin 
15 (IL-15) were purchased from Peprotech EC Ltd (London, UK). The 80-kd 
fibronectin fragment (FN80) was a gift of A. García Pardo (Centro de 
Investigaciones Biológicas, Madrid, Spain). A mixture of protease inhibitors 
(Complete Protease Inhibitors) was from Boehringer Mannheim Corp 
(Indianapolis, IN). Sodium orthovanadate was purchased from Sigma.

#### Cells

Neutrophils were isolated from fresh human blood by Ficoll-Hypaque 
density gradient centrifugation, which was followed by sedimentation at 1g 
in dextran (Sigma) at room temperature. The neutrophil-enriched fraction 
was further purified by hypotonic lysis of erythrocytes, which yielded a 
neutrophil-enriched fraction (300-400) and the uropod-bearing cells in 10 randomly selected fields 
(300-400) and the uropod-bearing cells in 10 randomly selected fields 
(60 × objective).

For double immunofluorescence analysis of neutrophils, fixed cells 
were incubated with the 38/87 mAb and then with a tetrachondamine 
isothiocyanate (TRITC)-labeled rabbit antiposome IgG. Subsequently, cover-

slips were saturated with normal mouse serum and incubated with 
FITC-conjugated TP1/24 anti-ICAM-3 mAb. In some experiments, neutrophils 
were incubated for 5 minutes with confluent monolayers of endothelial 
cells previously activated with TNF-α (20 ng/mL) for 4 hours. For 
double staining of endothelial cells and neutrophils, coverslips were 
incubated with anti-ICAM-1 mAb MEM 111 followed by TRITC-labeled rabbit 
antiposome IgG. Coverslips were then saturated with normal mouse serum and 
incubated with FITC-conjugated TP1/24 anti-ICAM-3 mAb. Cells were 
observed with 60 × and 100 × oil-immersion objectives and a photo-
microscope (Nikon Labophot-2; Nikon, Inc, Melville, NY). Images were 
recorded on Ektachrome 400 film (Eastman Kodak Co, Rochester, NY).

#### Complementary DNA fragments for expression of the cytoplasmic 
regions of ICAM-3 and PSGL-1 were obtained by polymerase chain reaction 
amplification and cloned as Shall-Orf fragments into pGEX-4T (Pharmacia 
LKB Biotechnology, Uppsala, Sweden). Primers for amplification of the 
PSGL-1 cytoplasmic region were SALCYPYSG5'-TCCGCGTCGAC- 
GCCTTCGTCAGGAGGAGCA3'-and NOTCYPYSG5'-ATAAGAATGCC-
GCCGCCTAAGGAGAGACCTGATCA3'; those for ICAM-3 were 
SALCY50TH 5'-CAACGGTCGACAGGGACCAACCAGG3' and 
NOTCY50TH 5'-ATAAGAATGCCGCCTACTAATCTA-
GCTCTGGA3'.

For expression of GST fusion proteins in DHOIB cells and 
proteins were visualized with use of silver staining. Fractions contain-
ing ICAM-3 were pooled and concentrated 30-fold by using microconcen-
trators (Cetricon-100; Amicon, Danvers, MA).

Concentrated fractions were subjected to 8% SDS-PAGE under reducing 
conditions and transferred onto a nitrocellulose membrane (Millipore 
Corp, Bedford, MA) in Tris-glycine-methanol buffer for 30 minutes at 17 V 
for using a transfer cell (Transfer-Blot SD Semi-Dry Transfer Cell; Bio-Rad 
Laboratories, Hercules, CA). To detect moesin or ezrin, membranes were 
blotted overnight in TBS containing 3% BSA, washed 3 times with TBS 
and 0.1% Tween 20 for 15 minutes, and incubated for 1 hour with a 1:1000 
dilution of the rabbit antimoesin pAb 95/2 or with a 1:4000 dilution of the 
rabbit antiezrin pAb 90/3. After 3 washes, blots were incubated with a 
peroxidase-conjugated goat antirabbit IgG (Pierce Montlucon), and pro-
teins were visualized with use of an enhanced chemiluminescence detection 
system (Amersham, Little Chalfont, UK).

### Purification of recombinant moesin and in vitro binding assay

Recombinant full-length moesin and the N-terminal domain of moesin 
containing amino acid residues 1 to 310 (pKgMSN) were described previously.\textsuperscript{40} 
Expression of GST-fusion proteins in DHOIB cells and 
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and isolated and purified as GST-fusion proteins as described previously.\textsuperscript{40,41} Cleavage of the fusion protein bound to glutathione-Sepharose 4B beads (Pharmacia LVB Biotech) preequilibrated with 50 U/mL of thrombin (Pharmacia LVB Biotech) in Tris buffer (50 mmol/L of Tris-HCl [pH 7.5] and 150 mmol/L of NaCl) occurred after shaking at room temperature for 12 hours. The supernatant was loaded onto a 0.8 × 4-cm column (Pharmacia LVB Biotech) preequilibrated with washing buffer (50 mmol/L of Tris-HCl [pH 7.5], 1 mmol/L of phenylmethanesulfonyl fluoride, and 4 μg of leupeptin); the column included 6 mL of heparin and Sepharose. Proteins were eluted by using a step gradient of 0.2 mol/L, 0.4 mol/L, 0.5 mol/L, and 1 mol/L of NaCl. All purification procedures were performed at 4°C. Fractions containing full-length and N-terminal moesin were pooled and dialyzed against Tris buffer (50 mmol/L of Tris-HCl [pH 7.5] and 150 mmol/L of NaCl). The amount of recombinant moesin obtained was estimated by SDS-PAGE with use of known amounts of BSA.

Recombinant moesin was assayed for binding activity by incubation with glutathione-Sepharose–linked GST-ICAM-3 and GST-PSGL-1 fusion proteins in binding buffer (150 mmol/L of NaCl, 20 mmol/L of Tris-HCl [pH 8.3] and 0.2% Triton X-100) at 4°C. After 1.5 hours, the beads were washed 6 times in binding buffer. GST fusion proteins and their associated proteins were eluted by using 50 mmol/L of Tris buffer containing 20 mmol/L of glutathione. Bound moesin was assessed by SDS-PAGE and immunoblotting using moesin 95/2 pAb.

In vitro translation and binding assay

The pCR3 plasmids carrying the N-terminal domain of moesin containing inserts of amino acid residues 1 to 310 (MSNn/pCR3) were described previously.\textsuperscript{40} These plasmids were transcribed and translated in vitro by using a TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of T7 RNA polymerase and methionine labeled with sulfur 35 (\textsuperscript{35}S), according to the manufacturer’s instructions. Isotope-labeled moesin was incubated in binding buffer (20 mmol/L of Tris-HCl [pH 8.3], 150 mmol/L of NaCl, and 0.2% Triton X-100) at 4°C with GST-ICAM-3 and PSGL-1 fusion proteins that had been immobilized on glutathione-Sepharose beads. After 1.5 hours, the beads were washed 6 times in binding buffer, resolved on SDS-PAGE, and subjected to autoradiography.

Results

Induction by chemoattractants of redistribution of adhesion receptors and moesin to the uropod in neutrophils

In response to chemoattractants, neutrophils begin to migrate and form uropods, and we decided to analyze this response in detail. ICAM-3 and other adhesion molecules were evenly distributed in the plasma membrane in unstimulated neutrophils (Figure 1i and data not shown). When treated with the chemokine IL-8, the adhesion molecules ICAM-3, PSGL-1, CD43, and CD44, but not CD45, redistributed to uropods. Other chemoattractants (fMLP and thrombin (Pharmacia LVB Biotech) in Tris buffer (50 mmol/L of Tris-HCl [pH 7.5] and 150 mmol/L of NaCl), resolved on SDS-PAGE, and subjected to autoradiography.

Coelution of ICAM-3 and moesin from human neutrophils

To determine whether ICAM-3 is associated with moesin in neutrophils, ICAM-3 was isolated from lysates by antibody-affinity chromatography. A protein of 78 kd eluted at approximately pH 11 and was partly separated from ICAM-3, which eluted at pH 11.5 as a broad band of nearly 125 kd (Figure 4A). When the pooled fraction containing ICAM-3 was analyzed by immunoblotting, moesin was detected (Figure 4B). This immunoreactive polypeptide corresponded to the 78-kd band on the gel (Figure 4A). The original lysate of neutrophils reacted with antibodies to both moesin and ezrin,\textsuperscript{34} although higher amounts of moesin than of ezrin were detected with specific antibodies (data not shown). Ezrin was not detected in the ICAM-3 fraction (Figure 4B). To exclude a
possible nonspecific effect, the pooled fractions were also analyzed in additional control experiments by immunoblotting with antibodies to GRP78, an immunoglobulin-binding and possible contaminating protein with a molecular mass similar to that of moesin. No reactivity was observed (data not shown).

Interaction of ERM proteins with the cytoplasmic region of PSGL-1

To investigate further the association between adhesion proteins and moesin, GST-fusion proteins of the cytoplasmic domains of PSGL-1 and ICAM-3 were prepared. Instead of neutrophils, HL-60 cells were metabolically labeled with $^{35}$S met-cys to search for labeled proteins in the lysate that would bind to the cytoplasmic domains of these 2 membrane proteins. Several radiolabeled polypeptides were recovered from the GST-PSGL-1– and ICAM-3–glutathione-Sepharose but not from control beads containing either GST or no ligand that included polypeptides of 75-78 kd (data not shown). In parallel experiments, lysates of unlabeled HL-60 cells were incubated with the GST-PSGL-1 cytoplasmic-domain fusion protein, and bound proteins were analyzed by Western blot assessment using moesin and ezrin antibodies. As shown in Figure 5, the cytoplasmic region of PSGL-1 bound 78- and 81-kd polypeptides that were identified as moesin and ezrin, respectively (Figure 5A). In contrast, none of the proteins bound to PSGL-1 corresponded to vinculin (Figure 5B).

Interaction of PSGL-1 and ICAM-3 with the amino-terminal domain of moesin

Several studies have shown that the binding of moesin and related proteins to membrane proteins requires additional factors that may include a conformational change to expose potential binding sites. When purified soluble recombinant full-length moesin or the isolated amino-terminal domain proteins were added to beads containing GST-fusion proteins of the cytoplasmic domains

Figure 2. Polarization of PSGL-1 in neutrophils migrating toward a chemoattractant gradient or on endothelial cells. (A) Neutrophils were allowed to adhere to FN80-coated coverslips for 10 minutes. C5a was then deposited at one edge of the bottom of the well (lower right-hand corner of photograph) and cells were allowed to migrate for 5 minutes. Cells were then fixed and stained with anti-PSGL-1 KPL-1 mAb (green fluorescence). Epifluorescence and bright-field conditions were photographed on the same frame by means of double exposure. (B) Neutrophils were allowed to adhere to an endothelial cell monolayer activated by TNF-α for 5 minutes at 37°C. Cells were then fixed and double stained with anti-ICAM-3 TP1/25 mAb (green fluorescence) and anti-ICAM-1 MEM111 (red fluorescence) mAb. (C) Neutrophils were allowed to adhere to an endothelial cell monolayer activated by TNF-α for 5 minutes at 37°C. Cells were then fixed and stained with anti-PSGL-1 KPL-1 mAb. Epifluorescence and bright-field conditions were photographed on the same frame by means of double exposure.

Figure 3. Redistribution of PSGL-1, ICAM-3, and moesin to the uropod of polarized neutrophils. Neutrophils adhering to FN80 were stimulated with IL-8 for 5 minutes. Cells were then fixed and stained. (A) ICAM-3. (B) PSGL-1. Inset shows colocalization of PSGL-1 (a) and ICAM-3 (b). (C) Moesin. Inset shows colocalization of moesin (c) and ICAM-3 (d).

Figure 4. Association of ICAM-3 and moesin in neutrophils. ICAM-3 was isolated from human neutrophil lysates by antibody-affinity chromatography. (A) A polypeptide of 78 kd (arrow) eluted in fractions at pH 11.0 (lanes 1-2) while ICAM-3 coeluted at pH 11.0 (lane 2) and separated from the 78-kd polypeptide at pH 11.5 (lanes 3-4). (B) Western blot analysis of purified recombinant ezrin (lane 1) and the pooled ICAM-3 fraction (lane 2) with ezrin antibodies and the same pooled ICAM-3 fraction (lane 3) and recombinant moesin (lane 4) with moesin antibodies. A strong signal for moesin showed that the 78-kd polypeptide coeluting with ICAM-3 (Figure 4A) corresponded to moesin.
of PSGL-1 and ICAM-3, we found only trace amounts of full-length moesin (Figure 6); however, there were much stronger signals with smaller immunoreactive polypeptides (~60 kd). In contrast to the weak binding observed for full-length moesin, the N-terminal domain of moesin bound strongly to the cytoplasmic domain of both PSGL-1 and ICAM-3 (Figure 6C). This result suggests that the bound immunoreactive polypeptides shown in Figure 6B represented enriched degradation products that contaminated the purified full-length moesin preparation.

The amino-terminal domain of moesin made in bacteria contains several additional amino acid residues that remain attached after cleavage by thrombin. To exclude a contribution of this additional structure to binding, we tested the same domain of moesin made by in vitro translation. The amino acid sequence of this fragment corresponded precisely to the sequence of moesin from amino acid residues 1 to 310. As shown in Figure 7, the radioabeled probe associated with PSGL-1 and ICAM-3 but did not bind to the control GST.

**Discussion**

During acute inflammatory responses, selectins mediate initial rolling of neutrophils along the endothelial surface. Free-flowing leukocytes can either be synthesize or presented by activated endothelial cells, activate leukocyte integrins. The signals generated through selectins, combined with those triggered by chemoattractants, result in integrin-mediated tight adhesion of leukocytes, followed by their transendothelial migration. Free-flowing leukocytes can also attach to and roll on monolayers of adherent leukocytes under hydrodynamic shear stress, and PSGL-1 can serve as the ligand for L-selectin in neutrophil-neutrophil interactions. The redistribution of PSGL-1, ICAM-3, and other adhesion molecules to the uropod may therefore enable activated cells to make contact with additional leukocytes and to increase neutrophil recruitment. The concentration of PSGL-1 in the uropod may also facilitate interaction with and recruitment of platelets.

L-selectin and other adhesion receptors involved in initiation of the leukocyte-endothelial cell adhesion cascade, such as PSGL-1, are concentrated in microvilli tips. Preferential localization in microvilli is thought to facilitate contact with the endothelium. Studies have shown that the uropod of leukocytes is highly enriched in microvilli and microspikes. This suggests that accumulation of adhesion molecules, such as PSGL-1, in the uropod of neutrophils stimulated by chemoattractants serves to capture other leukocytes.

Chemoattractants induce the full range of neutrophil activation events, including chemotaxis, oxygen free-radical production, and degranulation. An early event is a change in morphologic conformation and, as we observed in this study, polarization and redistribution to the uropod of several adhesion molecules and members of the ERM protein family, which link membrane F-actin. Activated and adherent neutrophils move on the endothelial surface and extravasate by migrating through endothelial clefs. Our data show that both crawling neutrophils and those migrating through a monolayer of endothelial cells have polarized morphologic features, with ICAM-3 and PSGL-1 redistributed to the uropod. These results concur with those of previous studies, which found that neutrophils stimulated by FMLP transmigrated across the endothelium and engaged other neutrophils through uropod-mediated contacts.

Protrusions of the plasma membrane in lymphocytes and neutrophils, described morphologically as microvilli or microspikes, have not been studied in detail. These structures, unlike microvilli on the apical surface of brush-border epithelia in the gut or kidney, do not contain villin or fimbrin. They are presumably more closely related to filopodia, microspikes, and retraction fibers of fibroblasts in culture.

It is well established that these micro-
extensions contain actin filaments and one or more members of a family of proteins (the ERM proteins) that include moesin, ezrin, and radixin. At least one of these proteins appears to be necessary for either the formation or maintenance of such microextensions.33 All 3 proteins bind F-actin at a site in the C-terminal domain that must be activated or unmasked, since no interaction has been observed with intact, undenatured full-length molecules.40,41,49

In cells, moesin, ezrin, and radixin form complexes with CD44, CD43, ICAM-1, ICAM-2, and ICAM-3,24,25,50-53 and direct low-affinity associations between recombinant full-length proteins and CD44 and ICAM-1 have been studied in vitro.7,25 The in vitro data in this study show that PSGL-1 and ICAM-3 bind poorly to full-length moesin but strongly to the isolated amino-terminal domain. This result is reminiscent of the strong interaction between actin filaments and the isolated C-terminal domain and may indicate that a conformational change is necessary to expose functionally important binding sites in the N-terminal domain. In the native configuration, N- and C-terminal domains of moesin associate in a manner that prevents F-actin binding.26-28,40,41 The same intramolecular association may prevent interactions with membrane proteins, as we observed in this study.

The binding of ICAM-3 and PSGL-1 to moesin from cell lysates but not from bacteria may be explained by the fact that, in neutrophils and HL-60 cells, a fraction of the moesin molecules can be structurally altered by phosphorylation or by formation of a complex with polyphosphoinositides. For example, phosphorylation by Rho-dependent kinase or protein kinase C disrupts the intramolecular association between N- and C-terminal domains, suggesting regulation by small GTPases of the Rho family and other signaling pathways.40,45,55 and cosedimentation of highly purified moesin from human platelets with F-actin was observed in vitro when the molecule was both phosphorylated at thyr558 and when it formed a complex with phospholipids.40,41 A study has found that tyrosine phosphorylation is required for the interaction between the N-terminal domain of ezrin and phosphatidylinositol 3-kinase,39 suggesting yet other mechanisms for activation. Although our in vitro binding data are consistent with this simple mechanism and a direct interaction between receptor proteins and moesin or ezrin, another possibility has not been excluded. Links between transmembrane proteins and the actin cytoskeleton in cells also develop by insertion of additional proteins containing the PDZ domain that interact with membrane proteins on one hand and moesin or ezrin on the other.57,58 This provides additional possibilities for regulation. Further work is required to determine whether ICAM-3 or PSGL-1 are among the receptors that use this mechanism.

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References


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