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Platelet moesin interacts with PECAM-1 (CD31)

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Platelet activation results in formation of filopodia and cell spreading by extension of lamellae. Moesin is a member of the ezrin/radixin/moesin (ERM) family of proteins, which localize in cell extensions like filopodia and function as cross-linkers between the actin cytoskeleton and the plasma membrane. Here we investigated whether the adhesion molecule PECAM-1 (CD31) is a membrane-binding partner for moesin in platelets. Our data show that moesin co-immunoprecipitated with PECAM-1 in lysates from thrombin-stimulated, but not resting platelets. Furthermore, PECAM-1 co-localized with moesin at the cell periphery and in filopodia of glass-activated platelets. Our observations suggest that moesin may play a role in platelet adhesion, linking PECAM-1 with the actin cytoskeleton.

Introduction

Blood platelets participate in the process of hemostasis and thrombosis. When platelets are activated they change shape converting from discs into spherical forms, followed by formation of filopodia and cell spreading by extension of lamellae.[1] Morphological changes require cytoskeletal rearrangements mediated and regulated by a variety of signal transduction events of cytoskeleton- and membrane-associated proteins.[2]

Ezrin/radixin/moesin (ERM) proteins are variably associated with cell surface protrusions, such as microvilli, filopodia, adhesion contacts and membrane ruffles.[3–9] The C-terminus of ERM proteins binds to actin filaments, whereas their N-terminus associates with several integral membrane proteins such as CD43 and CD44 and other adhesion molecules such as ICAM-1, -2 and -3[10–13] by a sequence of positively charged amino acid residues in their juxta-membrane cytoplasmic domain.[14–16] Cells contain soluble pools of ERM monomers which are dormant due to intramolecular association of their N- and C-terminal regions.[17,18] Phosphorylation at a C-terminal threonine residue, near an actin filament binding site, affects the direct interaction between the N- and C-terminus of radixin and ezrin, suggesting that this phosphorylation keeps ERM proteins in an open activated form.[19,20]

Although ezrin is the most broadly expressed ERM protein, moesin[21] is quantitatively dominant in leukocytes and is the only ERM protein expressed in platelets.[22] Activation of platelets by thrombin leads to a rapid phosphorylation of moesin on threonine 558 and its association with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2).[22,23] Moesin distributes to the periphery of the cell and in filopodia of glass-activated platelets.[22] A similar distribution has been shown in platelets activated by thrombin in solution, combined with its rapid association with the membrane cytoskeleton.[23,24] However, the membrane-binding partner of moesin in platelets is unknown. Its identification may be a valuable contribution to the understanding of the role of moesin in these cells.

PECAM-1 (CD31) is a 130-kDa adhesion molecule expressed on the surface of circulating platelets, monocytes, neutrophils and selected T cell subsets

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and at the endothelial cell intercellular junction.[25,26] PECAM-1 plays a role in initiating endothelial cell contact and may serve to stabilize the endothelial cell monolayer.[27] PECAM-1 clustering results in increased integrin-mediated adhesion of T cells, platelets and transfected cells.[28–30] PECAM-1 associates with the actin cytoskeleton of activated, but not resting platelets,[31] and is highly phosphorylated on tyrosine during platelet aggregation.[32–35]

In the present study, we investigated whether PECAM-1 is a membrane-binding partner for moesin in platelets. Moesin co-immunoprecipitated with PECAM-1 in lysates from thrombin-stimulated, but not resting platelets. Furthermore, PECAM-1 co-localized with moesin at the cell periphery and in filopodia of glass-activated platelets. Our observations suggest that moesin may play a role in platelet adhesion, linking PECAM-1 with the actin cytoskeleton.

Materials and methods

Generation of a chicken anti-moesin antibody

Moesin was isolated from human platelets by a modified method described originally for the isolation of ezrin from human placenta.[36] A polyclonal antibody against purified moesin was elicited in Leghorn Braun chicken and purified from egg yolk by a PEG precipitation method.[37]

Platelet preparation

Platelet isolation was carried out according to well-described methods.[1,38] Briefly, human blood from healthy volunteers, drawn into Aster-Jandl anticoagulant (69 mM citric acid, 85 mM sodium citrate and 110 mM glucose), was centrifuged at $160 \times g$ for 20 min. The platelet-rich plasma was gel-filtered through a Sepharose 2B column equilibrated and eluted with a solution containing 145 mM NaCl, 10 mM Hepes, pH 7.4, 10 mM glucose, 0.5 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgCl_2$ and 0.3% bovine serum albumin (BSA). The measured density of platelets was 5×10^8/ml. Platelets were allowed to rest by standing at 37°C for 30 min.

Immunofluorescence analysis

Platelets were stimulated at 37°C either in suspension with 1 U/ml human thrombin (Sigma, St. Louis, MO) for 3 min or by sedimentation on glass coverslips for 10 min. Activation was terminated by fixation with methanol for 10 min at 20°C. The suspension was centrifuged onto polylysine-coated coverslips. Coverslips were blocked with 3% BSA in phosphate-buffered saline (PBS). The chicken anti-moesin antibody was incubated at room temperature for 2 h at 1:500 in PBS containing 0.5% BSA and 0.5% Triton X-100. When necessary, double staining for PECAM-1 was also performed using a monoclonal antibody (Dako, Carpinteria, CA). Controls included pre-immune chicken yolk or normal mouse IgG, as appropriate. FITC-coupled anti-chicken IgG or TRITC-coupled anti-mouse IgG secondary antibodies were incubated 30 min at room temperature. The slides were mounted with 50% glycerol in PBS. Cells were examined by a Leitz Fluorescence microscope DMR-X 100×1.3 oil immersion objective and photographed on Kodak T-MAX 400 ASA films (Eastman Kodak, Rochester, NY).

Electron microscopy

Platelets were stimulated by sedimentation on glass coverslips for 10 min at 37°C, fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, and processed for scanning electron microscopy.

Immunoprecipitation, SDS–PAGE and immunoblot analysis

Platelet suspensions were activated by addition of 1 U/ml thrombin for 3 min at 37°C in a Bio/Data aggregometer Horsham, PA with or without constant stirring at 1000 rpm. Activation was terminated by addition of 0.5 volume of 3× lysis buffer (3% Nonidet P-40, 150 mM Tris–HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 3 mM Na_3VO_4, 30 μg/ml leupeptin and 30 μg/ml aprotinin), as described.[39] Insoluble fractions were sedimented by centrifugation at $14\,000 \times g$ for 10 min, and soluble fractions were immunoprecipitated with a monoclonal anti-PECAM-1 antibody (R&D Systems, Minneapolis, MN) bound to protein G-Sepharose beads. Immune complexes were solubilized in SDS–PAGE loading buffer containing 5% β-mercaptoethanol. After boiling for 5 min, proteins were separated by SDS–PAGE on an 8% polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated overnight in Tris-buffered saline containing 1% BSA and 0.05% Tween-20, then probed with anti-PECAM-1 or anti-moesin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Detection was performed with an enhanced chemiluminescence SuperSignal system (Pierce, Rockford, IL).

Results

Localization of moesin in resting and thrombin-stimulated platelets

Moesin, isolated and purified from human platelets, was used to obtain a specific antibody from chicken that is suitable for immunofluorescence studies. Immunoblot analysis showed a specific reaction

with the chicken anti-moesin antibody in lysates from human platelets and human umbilical vein endothelial cells (HUVECs) (Figure 1a). In HUVECs, an additional faint band of slightly slower motility could be discerned. This band could be attributed to radixin, using the monoclonal antibody 38/87,[7] kindly provided by Dr. Schwartz-Albiez (Heidelberg, Germany), which reacts with both radixin and moesin (data not shown).

Moesin localization in resting and in thrombin-stimulated platelets was studied by immunofluorescence with the chicken anti-moesin antibody and a FITC-coupled secondary antibody. In resting platelets, although some granular patterns could be discerned, a diffuse signal was seen throughout the cytoplasm without a prevalence of any specific intracellular compartment or structure (Figure 1b). Activation of platelets in solution with 1 U/ml thrombin induced an almost complete redistribution of moesin towards the cell periphery (Figure 1c).

Association of moesin with PECAM-1 in thrombin-stimulated platelets

ERM proteins interact with adhesion molecules like CD43, CD44 and ICAM-1, -2 and -3 by a sequence in their juxta-membrane cytoplasmic domain consisting of positively charged amino acid residues, notably arginine and lysine.[14–16] Figure 2 shows that the juxta-membrane cytoplasmic domain of PECAM-1 contains a positively charged sequence RKAKAK at position 599–604 similar to known membrane-binding partners of ERM proteins.

To determine whether PECAM-1 is associated with moesin in platelets, PECAM-1 was immunoprecipitated from lysates of resting and thrombin-stimulated platelets. Figure 3 shows that moesin co-immunoprecipitated with PECAM-1 in thrombin-stimulated, but not resting platelets. Platelet activation was performed with or without stirring to stimulate platelet aggregation, in conditions where tyrosine phosphorylation of PECAM-1 and its association with signaling proteins like SHP-1 and -2 have been described.[32–35] The presence or absence of stirring did not affect the intensity of the moesin band, suggesting that the association between moesin and PECAM-1 was independent of platelet aggregation. Moesin could not be directly immunoprecipitated in our conditions (data not shown). This may be attributable to the antibodies used and/or to the conformation changes of moesin that occur during platelet activation.

Co-localization of moesin with PECAM-1 in glass-activated platelets

We further investigated the localization of moesin and PECAM-1 in glass-activated platelets by immunofluorescence analysis (Figure 4a,b). PECAM-1 showed a pattern of staining identical to that observed for moesin, distributing especially at the cell periphery and in filopodia of glass-activated platelets (see encircled cells).[22] An enhancement of the fluorescent signal in the filopodial tips could be

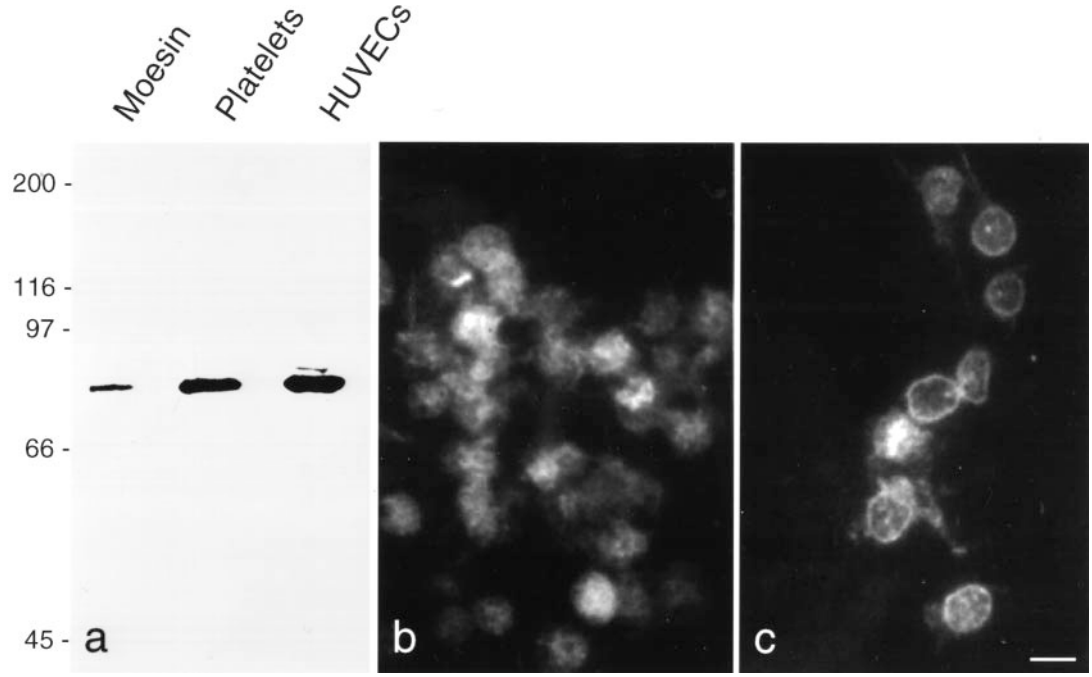

Figure 1. Localization of moesin in resting and in thrombin-stimulated platelets. Purified moesin, and whole platelet and HUVEC lysates were submitted to SDS–PAGE on an 8–25% gradient gel followed by transfer onto an Immobilon-P membrane, as indicated. Moesin was detected by immunoblot using the chicken anti-moesin antibody and a peroxidase-conjugated secondary anti-chicken antibody (a). Gel-filtered platelets were stimulated in suspension by addition of 1 U/ml thrombin for 3 min without stirring. Resting (b) and activated (c) platelets were stained with the chicken anti-moesin antibody and visualized with a FITC-conjugated secondary anti-chicken antibody. The bar is 5 μm.

CD43	259-278	**RRR**Q**KRR**TGALVLS**R**GG**KR**N
CD44	651-670	NS**RRR**CGQ**KKK**LVINSGNGA
ICAM-1	479-498	N**R**Q**RK**I**KK**Y**R**LQQAQ**K**GTPM
ICAM-2	228-247	Q**H**L**R**QQ**R**MGTYGV**R**AAW**RR**L
ICAM-3	481-500	**R**E**H**Q**R**SGSY**H**V**R**EESTYLPL
PECAM-1	594-613	**K**CYFL**RK**A**K**A**K**QMPVEMS**R**P

Figure 2. Amino acid sequence alignment of the juxta-membrane cytoplasmic domains of human CD43, CD44, ICAM-1, ICAM-2, ICAM-3 and PECAM-1. Sequences are available from SWISS-PROT (http://www.expasy.ch/sprot/) under accession number P16150, P16070, P05362, P13598, P32942 and P16284, respectively. Positively charged amino acid residues are indicated in bold.

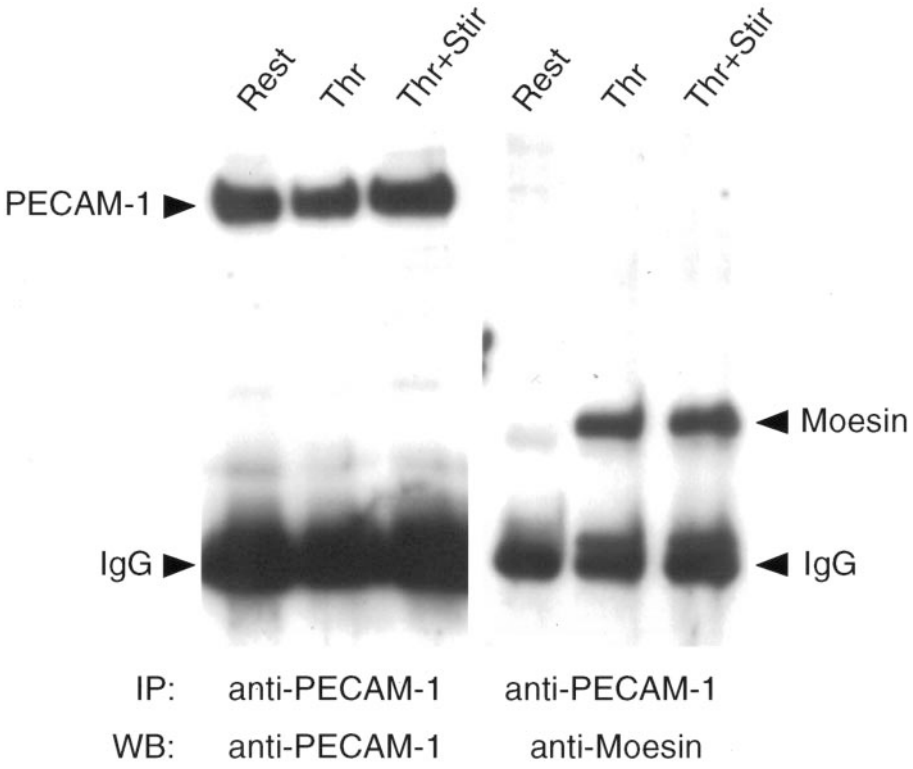

Figure 3. Moesin associates with PECAM-1 in thrombin-stimulated platelets. Platelets were resting (Rest) or stimulated in suspension by addition of 1 U/ml thrombin for 3 min at 37°C with (Thr+Stir) or without (Thr) stirring to promote aggregation. Platelets were lysed by addition of a Nonidet P-40-containing lysis buffer and PECAM-1 was immunoprecipitated. The immune complexes were separated by SDS–PAGE, transferred onto an Immobilon-P membrane and probed with anti-PECAM-1 and anti-moesin antibodies, as indicated.

observed in some cells. We further investigated the morphology of glass-activated platelets by scanning electron microscopy (Figure 4c). This revealed some of the filopodial ends as small flat structures (see arrows).

Discussion

In the present study, we have examined the localization of moesin in resting and in activated platelets and investigated its possible interaction with the adhesion molecule PECAM-1. The juxta-membrane cytoplasmic domain of PECAM-1 contains a cluster of positively charged amino acid residues at position 599–604 similar to known membrane-binding partners of ERM proteins such as CD43, CD44 and ICAM-1, -2 and -3 (Figure 2). Our data show that moesin co-immunoprecipitated with PECAM-1 in thrombin-stimulated, but not resting platelets (Figure 3). Furthermore, PECAM-1 localized at the cell periphery and in filopodia of glass-activated platelets where it co-localized with moesin (Figure 4a,b).

Moesin distribution at the cell periphery and in filopodial protrusions has already been described in glass-activated platelets and in platelets activated by thrombin in solution.[22,24] However, its membrane-binding partner was not identified. The interaction between moesin and PECAM-1 suggests that moesin may participate in PECAM-1 clustering to stimulate integrin-mediated platelet adhesion. Moesin, through linkage with the cytoskeleton, promotes cell adhesion by redistributing linked surface membrane adhesion molecules, notably CD44 and ICAM-3 to the uropods of polarized T cells.[12,13] Furthermore, PECAM-1 clustering results in increased integrin-mediated

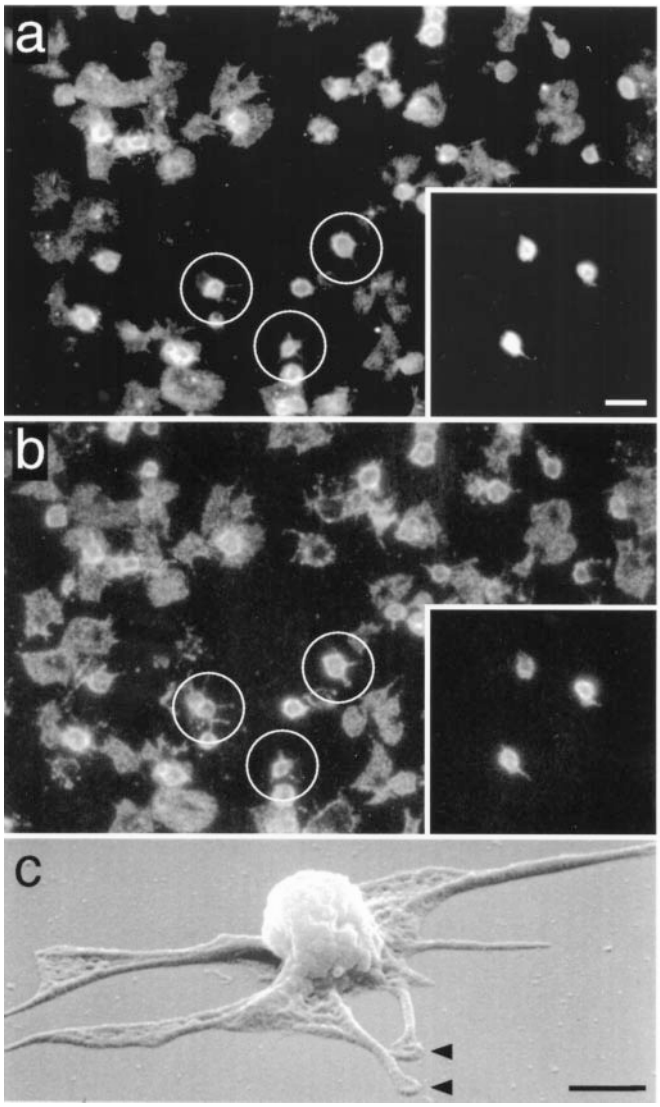

Figure 4. Moesin co-localizes with PECAM-1 in glass-activated platelets. Resting platelets (insets) were sedimented on glass coverslips for 10 min and double stained for moesin and PECAM-1. The staining for moesin was performed with the chicken anti-moesin and a FITC-conjugated secondary antibody (a). The staining for PECAM-1 was performed with a monoclonal anti-PECAM-1 antibody and a TRITC-conjugated secondary antibody (b). The bar is 10 μm. Representative morphology of a single glass-stimulated platelet by scanning electron microscopy (c). Platelets were stimulated by sedimentation on glass coverslips for 10 min at 37°C. The bar is 1 μm.

adhesion of T cells, platelets and transfected cells.[28–30] Moesin and PECAM-1 distributed especially at the cell periphery and in filopodia of glass-activated platelets, with an enhancement of the fluorescent signal in filopodial tips, which were revealed by scanning electron microscopy as small flat structures that may serve as adhesion sites (Figure 4c).

The regulation of the interaction between moesin and PECAM-1 is unknown. PECAM-1 is highly phosphorylated on tyrosine during platelet aggregation and acts as a negative regulator of the platelet responses to the collagen receptor GPVI/FcRγ-chain complex.[40,41] Earlier studies showed that phosphorylation of PECAM-1 on tyrosine 663 and 686 leads to binding and activation of the SH2 domain-containing protein tyrosine phosphatases SHP-1 and -2.[32–35] The association between PECAM-1 and moesin was independent of platelet aggregation, suggesting that PECAM-1 tyrosine phosphorylation is not required for its association with moesin.

On the other hand, moesin is rapidly phosphorylated on threonine 558 during platelet activation by thrombin, allowing it to bind actin filaments.[22] Moesin associates with the actin cytoskeleton of activated, but not resting platelets[23,24] (KMH, unpublished data), as does PECAM-1.[31] In activated platelets, moesin also binds PtdIns(4,5)P_2,[23] the synthesis of which increases downstream of the small GTPase Rac.[42] It has been shown that activation of the small GTPases Rac and Rho by GTPγS in permeabilized fibroblasts leads to moesin localization to focal adhesion sites by a mechanism that may require PtdIns(4,5)P_2 production.[43] Furthermore, PtdIns(4,5)P_2 stabilizes the association of ERM proteins with CD44 and ICAM-1 and -2 in transfected cells.[11,15] Thus, it is possible that the

interaction of moesin with PECAM-1 occurs downstream of moesin phosphorylation and PtdIns(4,5)P_2 binding in activated platelets.

In conclusion, our data show that moesin is associated with the adhesion molecule PECAM-1 during platelet activation and suggest that moesin may link PECAM-1 with the actin cytoskeleton. This interaction may play a role in the formation of adhesion sites leading to platelet shape change and activation.

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