Phosphorylation of Threonine 558 in the Carboxyl-terminal Actin-binding Domain of Moesin by Thrombin Activation of Human Platelets*

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The phosphorylation and localization of the membrane-linking protein moesin was analyzed during early activation of platelets with thrombin. Activated platelets elaborate filopodia and spread to assume flat pancake-like shapes, and moesin is localized in filopodia and cell body. In resting platelets, approximately 25% of moesin molecules are phosphorylated as shown by metabolic labeling with [32P]Pi and by isoelectric focusing. Within seconds after exposure to thrombin, phosphorylation increases, reaching a maximum of 35% labeled molecules by 1 min, followed by a decrease to a new basal level within 5 min. This modification affects a single residue, Thr558, which is located within or close to a binding site for F-actin. Rapid shifts (0–100%) in the number of phosphorylated molecules are observed in the presence of inhibitors of serine/threonine kinases and phosphatases. Inhibitors affecting tyrosine phosphorylation also modulate phosphorylation at this site suggesting that the enzymes involved in the modification of Thr558 are regulated by tyrosine phosphorylation. Platelets respond to both extremes of modification by forming extremely long filopodia and the absence of spreading on glass. Completely phosphorylated moesin is concentrated together with F-actin in the center of the cell. The rapid modification of moesin at or near its actin-binding domain suggests a model for regulated membrane-cytoskeleton interaction during cell activation.

Platelets are anucleate, membrane-bound particles that participate in the process of hemostasis and thrombosis. They can be activated by a wide variety of stimuli and predictably respond to such stimuli by changing shape, by releasing the content of storage granules, by adhesion, and by aggregation (1, 2). These phenomena require drastic changes in the organization of the cytoskeleton and thus make platelets a powerful event that mediates platelet cytoskeletal rearrangements have been shown to be largely mediated through phosphorylation and dephosphorylation of cytoskeleton-associated and membrane proteins (3, 4). Such modifications result in changes in protein-protein interactions and/or subcellular distribution and allow for the formation of new protein complexes between membrane components and the cytoskeleton. Following activation by physiological stimuli, for example, phosphorylation of moesin light chain, actin-binding protein, P-selectin, glycoprotein IIb, glycoprotein Ib, talin, vinculin, cofillin, pp60src, and focal adhesion kinase (pp125FAK) is induced and regulates some of the molecular changes involved in platelet aggregation and release (5–22).

Moesin is a member of the protein 4.1 family (23, 24) and shares considerable structural homology with ezrin and radixin (25–30). These proteins are often co-expressed in a great variety of cell types, where they are localized at the cytoplasmic face of the membrane in filopodia and other microextensions of the cell surface (31–34). Such microextensions are considered important for cell-cell and cell-substrate recognition, for signal transduction, and for motility (35). Moesin, ezrin, and radixin are hypothesized to provide reversible links between membrane components and the actin cytoskeleton in these specialized domains of the plasma membrane.

This model for moesin as a membrane-cytoskeleton linking protein was originally adapted from and is based on structural features of the cytoskeletal protein 4.1. In the human red cell, the amino-terminal domain of this protein, homologous to a domain in moesin, interacts with the transmembrane glycoprotein glycoporphin C, while a central 10-kDa region of the protein mediates binding to the spectrin-actin meshwork (36, 37). Membrane-binding sites are largely unknown for moesin, ezrin, and radixin, but recent direct in vitro binding studies demonstrated these proteins interacting with F-actin at a site in their highly conserved carboxyl-terminal domains (38, 39). Given that moesin is located in specialized microdomains of the membrane-cytoskeleton which are dynamic and involved in cell movement, and given that moesin can bind F-actin in vitro, how are these interactions regulated? Evidence for regulation comes from work showing that in gastric parietal cells, the homologous protein ezrin is phosphorylated at serine and threonine residues (40). Also, in A431 tumor cells, ezrin is a substrate for the EGF1 receptor tyrosine phosphokinase, and Tyr145 and Tyr353 have been identified as major phosphorylation sites (41), but serine and threonine residues are modified as well (42). In NIH 3T3 cells overexpressing EGF receptor and

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The abbreviations used are: EGF, epidermal growth factor; pAs, polyclonal antisera; pAb, polyclonal, affinity-purified antibody; BSA, bovine serum albumin; PGI2, prostacyclin; PGE1, prostaglandin E1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TEMED, N,N,N′,N′-tetramethylethylenediamine; RIPA, radioimmunoprecipitation assay; PAO, phenylarsine oxide; GST, glutathione S-transferase; DIC, differential interference contrast.

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radixin, differential tyrosine phosphorylation of ezrin and radixin was stimulated with EGF and platelet-derived growth factor, respectively. Although almost all tyrosine residues are conserved, no phosphorylation has been found for moesin (43).

We show in this study that in contrast to most other cells (35), only moesin and not ezrin or radixin is expressed in human platelets. It is phosphorylated on a single threonine residue and, in response to activation by thrombin and in the presence of inhibitors of serine/threonine and tyrosine phosphokinases and phosphatases, moesin phosphorylation is rapidly modulated.

MATERIALS AND METHODS

Antibodies and Cells—Mouse monoclonal antibody 38/87 was used for the identification of moesin and radixin in immunoblots (23). Rabbit polyclonal reagents pAEM and pAER refer to sera raised to purified human ezrin and recombinant human radixin (32, 35). They are specific for the respective proteins by immunoblotting. The affinity-purified polyclonal moesin-specific antibody pAEMoR was isolated from the antisera pAEMoR, as shown elsewhere (35). Mouse monoclonal antibody 4G10, specific for phosphorylated tyrosine residues, was purchased from UBI (Lake Placid, NY). The porcine kidney epithelial cell line LLC-PK, and megakaryoblast cell line K-562, were obtained from ATCC (Rockville, MD).

Isolation and Activation of Platelet Suspensions—Human blood was drawn into 0.15 volume of National Institutes of Health (NIH) formula A acid/citrate/dextrose solution and gently mixed. Platelet-rich plasma was prepared by centrifugation of whole blood at 200 × g for 15 min. To remove any contaminating erythrocytes and leukocytes, the plasma was layered onto a 40% sucrose cushion at 100 × g for 30 min. The resulting platelet-rich plasma was gel-filtered into HEPES-Tyrode’s buffer (136 mM NaCl, 2 mM CaCl2, 0.5 mM Na2CO3, 4.4 mM glucose, pH 7.4) as described previously (44). Fifty ng/ml PGL2 was added to the enriched platelet suspension. Prior to activation, platelets were centrifuged and resuspended in Tyrode’s buffer (136 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.36 mM Na2HPO4, 1.8 mM CaCl2, 0.4 mM MgCl2) to 1 × 108 platelets/ml. Platelets were activated by the addition of 1.0 NIH unit of thrombin/ml (Sigma). For some experiments, platelets were incubated for 10 min with 1 μM okadaic acid, 0.01–1 μM calpisin A, 0.1–10 μM calphosphin C, 37–740 μM genistein, 0.1–10 μM staurosporine, 1–10 μM cytochalasin D (Sigma), 1–100 μM PAO (Alrich), and 0.1–10 μM H-89 (Sakagaku America). Platelet aggregation was measured in a Chronolog Aggregometer (Model 440V, Haverton, PA), generously made available to us by Dr. L. Leung, Dept. of Medicine, Stanford University) with constant stirring at 100 rpm. All experiments were performed at 37 °C.

Differential Interference Contrast (DIC), Time-lapse Video, and Immunofluorescent Microscopy—To examine platelet adhesion in morphometry during activation and to localize moesin in platelets, we used an inverted microscope on a Zeiss Axiosvert 35 inverted microscope equipped with epifluorescence, DIC optics, Panasonic time-lapse video recorder, B&W video camera, and a Hamamatsu Argo 20 image processor. Platelet suspensions in Tyrode’s buffer at 37 °C with or without 1 μM staurosporine, 100 μM calpisin A, or 1 NIH unit/ml thrombin were placed into the chamber, and the platelets were imaged by DIC and digital contrast enhancement as they attach and spread on the glass surface. At different time points after activation, platelets were fixed by perfusing the chamber with paraformaldehyde-lysine-periodate fixative (35, 45). Platelets were monitored by videomicroscopy during fixation, and the subsequent staining procedure was performed on-stage. The permeabilization and blocking solution contained 1% saponin in PBS. 3% BSA, Affinity-purified rabbit moesin antibodies and FITC-labeled anti-rabbit IgG and rhodamine phalloidin (Sigma) were used for staining. After immunolabeling, mounting medium with 90% glycerol and 10 mM p-phenyleneediamine was perfused into the chamber, and the platelets were viewed under epifluorescence illumination. Selected video images were processed in a 8100/80AV PowerPC computer and processed with a sublimation printer. Resting platelets were fixed in solution by dilution into 10 volumes of paraformaldehyde-lysine-periodate fixative. To immobilize prefixed platelets, the chamber coverglass was coated with poly-l-lysine (Sigma), and platelets were allowed to attach before permeabilization and staining was done as described above. 32P-Labeling and Activation of Platelets—The gel-filtered platelets were resuspended at 1 × 108 cells/ml in incubation buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 10 mM HEPES, 5.5 mM glucose, pH 7.4). This suspension was incubated with 1 μCi/ml carrier-free 32P, (ICN Biomedicals, Irvine, CA) at 37 °C for 1 h, centrifuged at 800 × g for 15 min, and resuspended at 5 × 106 cells/ml in Tyrode’s buffer. Aliquots (200 μl) of the labeled platelet suspension were incubated at 37 °C with agonists. In some experiments, platelets were incubated for 10 min with inhibitors mentioned above prior to activation. After incubation for the specified time, the reaction was stopped by addition of lysis buffer.

Phosphoamino Acid Analysis—32P-Labeled or nonlabeled platelets were solubilized by the addition of 1.0 ml of ice-cold RIPA lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 5 mM EGTA, 20 mM molybdate, 0.1 mM trifluoroorpazamide, 5 mM sodium orthovanadate, 10 μM PAO, 40 μM sodium pyrophosphate, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 1 μM staurosporine, 100 μM calyculin A, pH 8.2), and insoluble material was removed by centrifugation at 15,600 × g for 3 min. In samples treated with thrombin, 0.2 μl/ml hirudin was included during the preparation to maintain all of the platelet moesin was then incubated with 20 μl of protein A-Sepharose (50% suspension in PBS) for 1 h at 4 °C to reduce nonspecific binding. The mixture was centrifuged at 15,600 × g at 4 °C, and the supernatant was combined with 10 μg of pAEMoR in 100 μl of PBS. To this solution 50 μl of protein A-Sepharose (50% suspension in PBS) was added. The suspension was shaken gently for 3 h at 4 °C, and the beads were washed three times with RIPA lysis buffer and twice with washing buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, pH 7.4), centrifuged at 15,600 × g, and resuspended in these buffers at 4 °C. The Protein A-Sepharose pellet was boiled in 50 μl of 1 × SDS sample buffer for 5 min, and the 1000 × g supernatant was applied to SDS-PAGE.

Two-dimensional Gel Electrophoresis—Isoelectric focusing was performed according to the method of O’Farrell (47) with some modifications (48). The samples were resuspended in a buffer containing 9.8 M urea, 2% (w/v) Nonidet P-40, 2% ampholyte, pH 8.9–8.5, and 100 mM dithiothreitol. Isoelectric focusing gels for the first dimension were cast in glass tubes (13 cm in length and 1 mm inside diameter) and contained 9.2 M urea, 4% acrylamide, 2% Nonidet P-40, 2% ampholyte (mixture of pH 6–8 and pH 3.5–10 (3:1, v/v)), 0.01% ammonium persulfate, and 0.07% TEMED. The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 60 min. Samples were loaded and focused at 400 V for 19 h and then at 800 V for 1 h. Proteins were separated in the second dimension by SDS-PAGE.

Phosphoamino Acid Analysis—32P-Labeled moesin was immunoprecipitated as described above (43). Solution changes in the SDS-PAGE, phosphopeptide moesin was electrophoretically transferred onto polyvinylidene difluoride membrane. The protein was visualized by Ponceau S staining and autoradiography and excised from the membrane. The membrane was washed with water and incubated with 200 μl of 5.7 M HCl for 2 h at 110 °C. The phosphoamino acids were resolved by electrophoresis at pH 3.5 on a cation exchange thin-layer plate, as described.

Sequence Analysis—The moesin molecules were isolated from the gel by digestion with CNBr, trypsin, endoproteinase Asp-N (Proteasomes fragi) as described previously (50). Phosphopeptides were separated on reversed-phase C8 (4.6 × 250 mm, Dynamax, Rainin) or C18 (4.6 × 250 mm, analytical, Vyde) columns using a gradient of water containing 0.1% TFA and acetonitrile containing 0.1% TFA and 0.1% trifluoroacetic acid at 0.5 ml/min. Radioactive fractions were either sequenced directly or further digested with proteases. Peptide sequencing was accomplished with an Applied Biosystem 475A gas phase sequencer.

RESULTS

Platelet Moesin—Moesin, radixin, and ezrin can be detected in whole lysates of LLC-PK1 cell line by immunoblotting with a mixture of antibodies specific for each protein (Fig. 1). The three antibodies migrate in 9% SDS-PAGE as bands of 78, 81, and 82 kDa, respectively. All three proteins are also expressed in the Dami megakaryoblast cell line, while in human platelets only moesin, but not radixin and ezrin, is found.

Platelets remained in the resting state during the isolation.
procedure, as indicated by their discoid morphology as well as staining with tubulin antibodies, which shows a characteristic ring-like distribution of microtubules. Moesin is distributed throughout the platelet, and, at the light microscopic level, immunofluorescence does not reveal association with any particular structure (Fig. 2A, D). As a result of activation, platelets alter their shape by forming filopodia and lamellipodia (Fig. 2A, I). In activated platelets, moesin is associated with the filopodia, but it is also distributed throughout the rest of the platelet. The moesin immunofluorescence signal is frequently enhanced at the tips of filopodia (Fig. 2A, B). The platelet in Fig. 2A formed several, approximately 3-μm-long, filopodial protrusions around its circumference. However, the final morphology of activated platelets varies widely and is dependent on the substrate (Fig. 2A, I and III). On BSA-coated glass slides, platelets develop numerous filopodia (Fig. 2I), but spread much less than those on uncoated glass during the same time period (not shown). Moesin is clearly codistributed with actin in delicate filopodia, as well as in thicker pseudopodia. When platelets are treated with thrombin and exposed to an uncoated glass surface, they respond rapidly with filopodial protrusion. In addition, they spread out over their filopodia by forming broad lamellae (Fig. 2A). On glass, no difference was seen either in the cell shape change response or in the distribution of moesin, whether or not platelets had been exposed to thrombin.

Phosphorylation of Moesin in Platelets—To determine whether moesin is phosphorylated, freshly isolated and washed platelets were metabolically labeled with 32P, lysed with RIPA buffer, and subjected to immunoprecipitation with pAbMo. A relatively small, but reproducible, amount of 32P incorporation into moesin was found (Fig. 3). Because phosphorylation and/or dephosphorylation could occur after lysis of the cells, inhibitors of phosphatases (100 mM calyculin A, 20 mM molybdic acid, 40 mM sodium pyrophosphate, 10 μM PAO, and 5 mM sodium orthovanadate) and/or kinases (1 μM staurosporine) were added to the RIPA buffer before lysis. As expected, phosphatase inhibitors consistently increased the level of radioactivity associated with moesin. Phosphorylation must have occurred within intact cells and is accomplished before lysis, since no phosphorylation was seen, when lysed platelets were incubated with γ[32P]ATP, γ[32P]GTP, or 32P, in the presence of the inhibitors (data not shown).

To evaluate the kinetics and extent of phosphorylation of moesin during platelet stimulation, 32P-labeled platelets were activated with 1 unit/ml thrombin for various times, lysed, and subjected to immunoprecipitation. The relative amount of 32P incorporation was measured by densitometry of autoradiograms after SDS-PAGE gels. As shown in Fig. 4, there was a rapid rise in phosphorylation within seconds of exposure to thrombin, whether or not platelets had been exposed to thrombin. The changes in shape due to activation of the platelet after attachment to glass were observed by DIC microscopy for several minutes (A), followed by fixation and staining with moesin antibodies (B). Note the filopodial localization of moesin in B, with enhancement of the fluorescent signal in filopodial tips. Unactivated platelets (C) were fixed in solution, attached to poly-L-lysine-coated coverslips, and stained with antibodies to moesin (pAbMo) (D) or tubulin (E). The subcortical ring of tubulin staining is characteristic for resting platelets. II, platelets were allowed to attach to glass and were observed by DIC from the initial time of attachment. The arrows point to the same location in each field. The platelet near the left arrow attached at about 1 min and underwent a phase of filopodial elongation, followed by lamellar or pseudopodial transformation of the filopodia (shown in A-C at 9.7, 14, and 17.5 min). At 17.5 min, the preparation was fixed and stained with moesin antibodies (C). In this platelet, moesin is distributed throughout the spreading platelet. The right arrow is near a platelet that was recorded for about 9 min before fixation and was fixed in the filopodial phase of activation. Moesin is present in growing filopodia as well as in the center of the platelet. The platelet below the arrow to the right attached last and was viewed for 3 min. It also contains moesin throughout, including the processes that are beginning to form. III, platelets activated by thrombin. The sequence begins 1.5 min after attachment of the platelet marked by the arrows (0 min, A). The other platelets had attached 9 and 4 min earlier. After an additional 12 min of observation, the preparation was fixed on stage. Fixation produced no change in the morphology of these platelets (B). Fixation was followed by staining with moesin primary and fluorescein isothiocyanate-labeled secondary antibodies (C) and rhodamine-phalloidin (D). Moesin and actin staining is seen in filopodia and cell bodies. Note the differences in scale between I, II, and III.

FIG. 1. Immunoblotting analysis of platelet proteins. Coomassie Blue-stained gels of whole lysate of human platelets (lane 1), Dami cells (lane 2), and LLC-PK₁ cells (lane 3) were separated by PAGE; corresponding immunoblot profiles (lanes 4-6, respectively) were obtained with a mixture of polyclonal antibodies raised against ezrin (pAbE, 1:500), moesin (affinity-purified pAbMo, 1:1000), and radixin (pAbR, 1:500). In comparison to Dami and LLCPK₁ cells, which express all three proteins, platelets contain only moesin.

FIG. 2. Differential interference contrast (DIC) and immunofluorescence microscopic localization of moesin in resting and thrombin-activated platelets. To observe activation and intracellular localization of moesin directly, freshly isolated platelets were allowed to attach to a glass coverslip previously coated with 3% BSA. Axial compression of filopodia induces a ring-like distribution of microtubules. Moesin is clearly codistributed with actin in delicate filopodia, as well as in thicker pseudopodia. When platelets are treated with thrombin and exposed to an uncoated glass surface, they respond rapidly with filopodial protrusion. In addition, they spread out over their filopodia by forming broad lamellae (Fig. 2A). On glass, no difference was seen either in the cell shape change response or in the distribution of moesin, whether or not platelets had been exposed to thrombin. To observe activation and intracellular localization of moesin directly, freshly isolated platelets were allowed to attach to a glass coverslip previously coated with 3% BSA. Activation begins 1.5 min after attachment of the platelet marked by the arrows (0 min, A). The other platelets had attached 9 and 4 min earlier. After an additional 12 min of observation, the preparation was fixed on stage. Fixation produced no change in the morphology of these platelets (B). Fixation was followed by staining with moesin primary and fluorescein isothiocyanate-labeled secondary antibodies (C) and rhodamine-phalloidin (D). Moesin and actin staining is seen in filopodia and cell bodies. Note the differences in scale between I, II, and III.
Phosphorylation of Platelet Moesin

Fig. 3. The effect of kinase and phosphatase inhibitors on the phosphorylation of moesin during immunoprecipitation. 32P-Labeled platelets were lysed with RIPA buffer containing a mixture of protein phosphatase (PP) inhibitors (200 mM calyculin A, 40 mM sodium pyrophosphate, 20 mM molybdate, and 5 mM sodium orthovanadate, and 1 mM PAO) and/or protein kinase (PK) inhibitor (1 mM staurosporine), and moesin was immunoprecipitated with pAb Mo by incubation for 3 h. Phosphorylated moesin was quantitated by SDS-PAGE, followed by autoradiography and densitometric analysis. The data are the means ± S.D. of three separate experiments.

Fig. 4. Time course of phosphorylation of moesin in human platelets in response to thrombin. 32P-Labeled platelets were incubated with 1 unit/ml thrombin at 37 °C. At various time points, the platelets were lysed with RIPA buffer containing protein phosphatase and protein kinase inhibitors as in Fig. 5, moesin was immunoprecipitated with pAb Mo, and peptides were resolved by SDS-PAGE on a 9% gel, followed by autoradiography and Western blotting. The relative amount of 32P incorporation into moesin was quantitated by densitometric analysis of the autoradiogram shown in the top part of the figure. The autoradiogram is representative of four separate experiments. Molecular mass standards are shown on the left. The data represent the means ± S.D. of seven determinations and are representative of four separate experiments with activated platelets (filled circles). The control values (open circles) are the means of two separate experiments. No difference was found in the amount of moesin as determined by Western blotting, and the increase in phosphorylation was significantly different from resting levels (p < 0.005) as determined by the paired t-test.

thrombin (−1.6-fold). Maximum phosphorylation was reached after 1–2 min, and phosphorylation levels decreased thereafter to near basal levels within 5 min.

Effects of Phosphatase and Protein Kinase Inhibitors on Moesin Phosphorylation and Immunodetection—To determine whether any of the known phosphatase inhibitors changed the phosphorylation of moesin, 32P-labeled platelets before thrombin stimulation were preincubated with okadaic acid and calyculin A as inhibitors of protein phosphatase types 1 and 2A (51, 52) and PAO as an inhibitor of protein-tyrosine phosphatase (53). As shown in Fig. 5, the phosphorylation of moesin was increased significantly by calyculin A. Although not as pronounced, okadaic acid and PAO also increased phosphorylation. This effect was observed for both unstimulated and stimulated platelets and was dose-dependent. Moreover, the increase in phosphorylation seen in the presence of protein phosphatase inhibitors was considerably higher than that induced by thrombin alone.

The effect of protein kinase inhibitors on moesin phosphorylation is shown in Fig. 6. Calphostin C (54), genistein (55), and H-89 (56) are specific inhibitors of protein kinase C, protein-tyrosine kinase, and cAMP-dependent protein kinase (protein kinase A), respectively, while staurosporine has broad specificity for inhibiting protein kinases (57–59). Genistein and staurosporine prevent the increased phosphorylation induced by thrombin in a dose-dependent manner, as well as reducing basal phosphorylation in unstimulated platelets. Phosphorylation was not affected by calphostin C or H-89.

In view of previous results on the phosphorylation of tyrosine residues in ezrin and radixin (41, 43), we examined whether the phosphorylation of moesin is due to modification of tyrosine residues. Phosphotyrosine antibodies, however, did not detect this modification in immunoprecipitated moesin isolated from resting and thrombin-activated platelets. In previous reports, it was also shown that preincubation of platelets with PAO or pervanadate (60) resulted in a marked increase in the degree of tyrosine phosphorylation of a number of different platelet proteins. We repeated this experiment, but, even under such conditions, no phosphorylation of tyrosine in moesin was seen, although obviously many other polypeptides are modified (data not shown).

Phosphoamino Acid Analysis—To determine which amino acids on moesin are phosphorylated, moesin was isolated from 32P-labeled platelets and then subjected to phosphoamino acid analysis. Phosphorylation occurred exclusively on threonine residues in both unstimulated and stimulated platelets (Fig. 7). Interestingly, calyculin A and the protein-tyrosine phosphatase inhibitor PAO significantly enhanced threonine phosphorylation of moesin, but again no tyrosine or serine phosphorylation was detectable.

Quantitation of Moesin Phosphorylation by Two-dimensional Gel Electrophoresis—Immunoprecipitated moesin from resting platelets could be separated by two-dimensional gel electrophoresis into four protein species that were detectable by immunoblotting with a and b as the major species (Fig. 8). After stimulation, an increase in peptides c and d with a more acidic pI of ~6.2 is quite apparent. To determine which of these isoforms was phosphorylated, 32P-labeled immunoprecipitated moesin from thrombin-treated platelets was analyzed. Only two of the four peptides were labeled and corresponded to the more acidic forms (Fig. 8C). Not shown here are results from experiments using inhibitors, which confirmed this interpretation. Staurosporine treatment yields only spots a and b, while calyculin A produces a complete shift to the more acidic forms. Recombinant moesin, produced in Escherichia coli, on the other hand, migrates to a position between spots a and b. This slight shift in mobility is probably caused by the absence of glycosylation in the recombinant form. Although this was not analyzed for, platelet moesin presumably is glycosylated similarly to moesin isolated from smooth muscle, which contains glucosamine (23).

Densitometry scanning of the two-dimensional immunoblot
estimated the acidic moesin isoforms from unstimulated platelets to account for 24 ± 6% (n = 3) of total moesin. After thrombin stimulation, the more acidic moesin isoforms increased to 35 ± 6% (n = 3) of total moesin with a mean increase of 1.5 ± 0.2-fold (n = 3). This increase was consistent with the observed incorporation of 32P into moesin as a result of thrombin activation (Fig. 4).

**Identification of Threonine Phosphorylation Sites**—As mentioned above, the addition of calyculin A resulted in only phosphorylated moesin isoforms, while staurosporine completely eliminated these forms. To localize sites of phosphorylation, 32P-labeled immunoprecipitated moesin from resting platelets was digested with trypsin, and the peptides were separated by high performance liquid chromatography on a C18 column. The peptide maps were identical, and two radioactive peaks eluted at 58.1% (1500 cpm) and 59.8% (4800 cpm) of 70% acetonitrile. Incubation with trypsin for 48 h increased the ratio.

**Phosphoamino Acid Analysis of Human Platelet Moesin**—[32P]Moesin was immunoprecipitated from resting (lane 1), thrombin-treated (lane 2), calyculin A- (lane 3), or PAO- (lane 4) treated platelets and separated by SDS-PAGE. Moesin was subjected to partial amino acid hydrolysis followed by high voltage electrophoresis, pH 3.5, on cellulose plate. The positions to which phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and free inorganic phosphate (Pi) migrated are indicated. The standards were detected with ninhydrin (not shown), and 32P-phosphoamino acids were subsequently revealed by autoradiography of the same cellulose plate.

**Separation of Moesin Isoforms by Two-Dimensional Gel Electrophoresis**—Moesin was immunoprecipitated from resting (A), thrombin-treated (B), or 32P-labeled (C) platelets. Platelets were lysed in RIPA buffer containing protein kinase and protein phosphatase inhibitors. The lysates were immunoprecipitated with pAbMo, and the immunoprecipitated proteins were analyzed by isoelectric focusing followed by SDS-PAGE. The pl values for the most basic and acidic isoforms were 6.5 and 6.2, respectively. The data in C are obtained by scanning on a PhosphorImager.

**Fig. 5.** The effect of phosphatase inhibitors on moesin phosphorylation. 32P-Labeled platelets were incubated with the protein phosphatase inhibitors okadaic acid (OA), calyculin A (CA), or phenylarsine oxide (PAO), at 37 °C for 10 min before stimulation with 1 NIH unit/ml thrombin. Platelets were lysed, and moesin was immunoprecipitated with pAbMo. Phosphorylated moesin was visualized by autoradiography after SDS-PAGE, and the degree of phosphorylation was quantitated by densitometric analysis. The data presented on the left are the means ± S.D. of three separate experiments. The data on the right are from a single experiment except for none, 10 μM okadaic acid, 100 nM calyculin A, and 10 μM PAO, which were incorporated from the left graph. Note the difference in scale between the left and right graph.

**Fig. 6.** The effect of protein kinase inhibitors on moesin phosphorylation. 32P-Labeled platelets were incubated with the protein kinase inhibitors calphostin C (CaC), genistein (Gen), staurosporine (Stau), or H-89 at 37 °C for 10 min before stimulation with 1 NIH unit/ml thrombin. Platelets were then lysed, and moesin was immunoprecipitated with pAbMo. Phosphorylated moesin was visualized by SDS-PAGE followed by autoradiography. The open bars refer to resting platelets, and the filled bars to platelets 1 min after thrombin activation. The degree of moesin phosphorylation was quantitated by densitometric analysis. The data on the left are the means ± S.D. of three separate experiments. The data on the right are from a single experiment except for none, 1 μM calphostin C, 370 μM genistein, 1 μM staurosporine, and 1 μM H-89 that were repeated three times.

**Fig. 7.** Phosphoamino acid analysis of human platelet moesin. [32P]Moesin was immunoprecipitated from resting (lane 1), thrombin-treated (lane 2), calyculin A- (lane 3), or PAO- (lane 4) treated platelets and separated by SDS-PAGE. Moesin was subjected to partial amino acid hydrolysis followed by high voltage electrophoresis, pH 3.5, on cellulose plate. The positions to which phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and free inorganic phosphate (Pi) migrated are indicated. The standards were detected with ninhydrin (not shown), and 32P-phosphoamino acids were subsequently revealed by autoradiography of the same cellulose plate.

**Fig. 8.** Separation of moesin isoforms by two-dimensional gel electrophoresis. Moesin was immunoprecipitated from resting (A), thrombin-treated (B), or 32P-labeled (C) platelets. Platelets were lysed in RIPA buffer containing protein kinase and protein phosphatase inhibitors. The lysates were immunoprecipitated with pAbMo, and the immunoprecipitated proteins were analyzed by isoelectric focusing followed by SDS-PAGE. The pl values for the most basic and acidic isoforms were 6.5 and 6.2, respectively. The data in C are obtained by scanning on a PhosphorImager.

**Fig. 9.** The effect of phosphatase inhibitors on moesin phosphorylation. 32P-Labeled platelets were incubated with the protein phosphatase inhibitors okadaic acid (OA), calyculin A (CA), or phenylarsine oxide (PAO), at 37 °C for 10 min before stimulation with 1 NIH unit/ml thrombin. Platelets were lysed, and moesin was immunoprecipitated with pAbMo. Phosphorylated moesin was visualized by autoradiography after SDS-PAGE, and the degree of phosphorylation was quantitated by densitometric analysis. The data presented on the left are the means ± S.D. of three separate experiments. The data on the right are from a single experiment except for none, 10 μM okadaic acid, 100 nM calyculin A, and 10 μM PAO, which were incorporated from the left graph. Note the difference in scale between the left and right graph.
Phosphorylation of Platelet Moesin

Moesin (Human) 550 RLGRDKYK;ROIQNGTKMRDEFESEM 577
Ezrin (Human) 560 GGRDKYK;ROIQNGTPQHDFEAL 586
Radixin (Human) 555 VGRDKYK;ROIQNGTKMRDEFESEM 583
EM10 (E. mul.) 534 GNDKVKYK;NIKGNMCTYQVEQESFM 599
MHC (E. his.) 1890 LQDQYKLY;VKGSVDFSQIQEMEQL 1915 ---
Merlin (Human) 569 GSGSSKHKYK;TLGSGSBVMAFEEL 595
CapZ (Chiken) 252 NQYKVLQ 260 ---
MHC-cm (Human) 719 RQVRLNP 727 ---
MHC-fsm (Rabbit) 719 KQRYKVLNA 727 ---

Fig. 9. Sequence comparison of the KYKX motif for moesin-like and actin-binding proteins. Underlined amino acids indicate sequence homology to moesin. Conserved threonine residue in the KYKX motif is indicated in **boldface italic**. MHC, myosin heavy chain; MHC-cm, myosin heavy chain from cardiac muscle; MHC-fsm, fast skeletal muscle; E.mul., Echinococcus multilocularis; E.his., Entamoeba histolytica; CapZ, actin capping protein.

of the second to the first radioactive peak, suggesting that the peptide in the first peak had been digested further. Radioactive peptides from only the calyculin A-treated platelets were analyzed. Each radioactive fraction was sequenced directly. The first fraction contained peptide Lys555-Arg557, which has one threonine in position 66 (24). Yields in the first and third cycle were 65 pmol of lysine and 39 pmol of threonine, respectively, suggesting that Thr558 is not phosphorylated. The first four cycles of the second fraction revealed residues DKYK, while the fifth cycle showed no signal and was followed by amino acids LR (Fig. 9). This sequence corresponded to fragment Asp554-Arg557. The lack of signal in the fifth cycle suggested that Thr558 could have been phosphorylated. To support this conclusion, we digested a second preparation of moesin with endoproteinase Asp-N, followed by endoproteinase Arg-C. Two radioactive fractions were again found at 18.6% (8,000 cpm) and 25.7% (11,000 cpm) of 70% acetonitrile on the same C18 column. Sequence analysis showed the first fraction to contain a mixture of three peptides, Asp554-Lys-Tyr-Lys-Arg-Leu-Ary-Glu-Ile-Arg563, Thr558-Ala-Met-Ser-Thr-Phe-His-Val-Ala563, and Glu570-Arg-Ileu-Gln-Val174, containing the three threonine residues Thr558, Thr569, and Thr570. However, we could tentatively exclude phosphorylation at two of these on quantitative grounds, since the first cycle gave 21 pmol of threonine (Thr558) and 11 pmol of aspartic acid, and the fifth cycle, expected to contain Thr558 and Thr567, yielded 16 pmol of threonine. This very likely excluded Thr558 and possibly also Thr567. In the second fraction, the initial four cycles of sequencing again identified residues DKYK, the fifth cycle showed no signal, and the remaining cycles yielded the sequence LRQR with reasonable yield (Fig. 9). This sequence included Asp554-Arg557 of the trypsin-digested peptide and, again, the complete lack of signal at the fifth sequencing cycle suggests, therefore, complete phosphorylation of Thr558. Finally, CNBr digestion was performed and a single radioactive fraction was eluted from the C18 column at 50.2% with 70% acetonitrile. The initial eight sequencing cycles of this peptide identified residues RLGRDKYK (Fig. 9), no signal was obtained for the ninth cycle, and Leu was found with high yield in position 10. This sequence corresponds to the CNBr peptide Arg550-Met557, and, although this fragment contains two threonine residues, Thr558 and Thr567, we conclude from the data described above that Thr558 constitutes the phosphorylation site of moesin, which is utilized during thrombin activation of platelets in vivo.

Effect of Inhibitors of Kinases, Phosphatases, and Actin Polymerization on Platelet Shape, Aggregation, and Subcellular Localization of Moesin—One well recognized event during platelet activation and aggregation is an increase in the content of F-actin and reorganization of actin filaments within the cell. To evaluate the importance of these changes for moesin phosphorylation, platelets were incubated with 2 mM cytochalasin D (61), an inhibitor of actin polymerization. No significant inhibition of moesin phosphorylation and platelet aggregation in response to thrombin stimulation could be detected (p > 0.3) (data not shown).

Platelet activation by agonists such as ADP, collagen, or thrombin leads to rapid aggregation in stirred suspensions. In an attempt to correlate the biochemical effects on moesin phosphorylation that we have observed in the presence of inhibitors of kinases, phosphatases, and actin polymerization, agents which affect the organization and integrity of the cytoskeleton and platelet function, we measured platelet aggregation by using an aggregometer. Calphostin C (0.1–10 μM) and H-89 (0.1–10 μM), as well as cytochalasin D (1–10 μM), did not produce measurable changes, whereas PAO (≥10 μM) and genistein (≥370 μM) treatment resulted in complete inhibition. Calyculin A (0.1–1 μM) and okadaic acid (1–10 μM) delayed aggregation, but aggregation reached control values within 5 min. A concentration of 1 μM staurosporine, on the other hand, partially inhibited the aggregation induced by thrombin by 55% (data not shown).

To determine how staurosporine and calyculin A, which completely inhibit or saturate phosphorylation of moesin, affect moesin and actin distribution, we analyzed platelet morphology directly by DIC microscopy and by immunofluorescence staining. As shown in Figs. 10 and 11, thrombin activation of both staurosporine- and calyculin A-treated cells resulted in platelets with very long filopodia. Staurosporine-treated cells, in fact, begin to protrude long filopodia even before they attach to the glass surface. This is most easily observed in A and B, where the rightmost arrow points to a filopodium on a platelet that had just landed on the surface and that begins to rotate forward as it lands. This platelet then continues to make long, thin and also branching filopodia. There is very little spreading at a time when normal platelets have already spread (Fig. 10E), although after 45 min some spreading is observed.

When platelets are then fixed and stained with antibodies to moesin, specific immunostaining is distributed throughout the platelet, including filopodia at both early (15 min, not shown) and late time points (46 min, Fig. 10G).

Calyculin A-treated cells, on the other hand, produce very long filopodia, some of which are more than 10 mm in length (Fig. 11). After an initial rapid phase of extension that lasted between 10 and 20 min, the filopodia do not continue to elongate and platelets do not spread. The distribution of moesin and actin is strikingly different from that of staurosporine-, restimulating, or thrombin-treated cells in that both proteins appear to be concentrated in the center. Moesin immunofluorescence appears as a ring or cylindrical protrusion in the center of the platelets. While a fluorescence signal for moesin is virtually absent from filopodia of calyculin A-treated cells, a low signal is seen with phalloidin in these structures.

**DISCUSSION**

This report is the first demonstration of a site-specific phosphorylation of moesin that is associated with cell activation. We have chosen the platelet for this study, because of an abundance of information on molecular events involved and because platelets express moesin as the only protein of several close homologues that are commonly found in other cell types. Platelets are derived from megakaryocytes by shedding from elongated membrane structures (62). In the Dami megakaryoblast cell line, at least three of the moesin-related proteins are expressed, but the expression pattern of megakaryocytes in the...
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bone marrow remains uncertain, because of the possibility that
expression of these proteins changes as cells adapt to tissue
culture.

The major conclusions of the present study are that moesin is
phosphorylated at a single threonine residue in resting and
thrombin-activated platelets and that phosphorylation at this
site is regulated by both protein phosphatases and kinases. In
comparison to resting platelets, phosphorylation at Thr558 in-
creases 1.6-fold within seconds after addition of thrombin and
returns close to the initial level within 5 min. These rapid
changes are not unique for moesin, since P-selectin (7, 8),
actin-binding proteins (6), and myosin light chains (5, 6) are
also rapidly phosphorylated with maximum phosphorylation at
30–60 s. Similar to our observations for moesin, phosphoryla-
tion decreases with time. The phosphorylation of glycoprotein
IIla reaches a maximum at 3 min, followed by a steady de-
crease (11). As shown by immunoblotting with anti-phosphoty-
rosine antibodies, phosphorylation of platelet proteins in-
creases in three distinct temporal waves after thrombin
activation (63): a rapid, but transient first peak within 5–20 s,
a second more sustained wave with a peak at 1–3 min, and a
third wave requiring longer incubation times (>3 min). The
increase in phosphotyrosine, phosphoserine, and phospho-
threonine levels is comparable to the increase seen with moesin
at 1.3–1.8-fold, 1.6–1.8-fold, and 1.8–2.4-fold, respectively (63).

Whereas many of these proteins are modified at multiple
sites (7), the modification of a single threonine in platelet
moesin is unique, and the rapid rise in the number of phospho-
ylated moesin molecules accompanying thrombin treatment is
highly significant. Moesin can be grouped together with other
first wave protein substrates, because of similar phosphoryla-
tion patterns. Phosphorylation of these proteins is thought to
reflect an early stage reaction of the platelet to the stimulus
by engaging cytoskeletal proteins necessary for shape change and
secretion. Dephosphorylation of moesin, on the other hand,
could be equally important for regulating such changes. Be-
cause phosphorylation and dephosphorylation of moesin occur
so rapidly after thrombin stimulation, it is difficult to clearly
delineate the kinetics of these changes. For example, we may
be underestimating the peak level of phosphorylation that oc-
curs within a single platelet because of the difficulty in har-
vesting platelet lysates within seconds of activation and be-
cause the platelet population may not be entirely synchronized
in its response during the initial few minutes. This may ac-
count for discrepancies in some of our experimental data. For
instance, the increase in phosphorylation in the presence of 100
nM calyculin A in three separate experiments ranged from
approximately 3–8-fold, while it was 6-fold for the single ex-
periment conducted with 1 μM (Fig. 5). Nevertheless, our esti-
mates for the number of phosphorylated molecules in resting,
thrombin-activated, and phosphatase-treated platelets are quite consistent and are based on data obtained by different methods as shown in Figs. 3, 4, and 8.

Peptide mapping and sequence analysis experiments clearly revealed Thr$^{558}$ as the only phosphorylation site in moesin. Previous studies on the related protein ezrin implicated phosphorylation of serine in histamine-stimulated gastric parietal cells, while in T cell receptor-activated lymphocytes from the lp strain of mice or the human J urkat T cell line, phosphorylation of tyrosine residue(s) is increased (40, 43, 64). The phosphorylation of threonine and tyrosine residues of ezrin is also increased by EGF in A431 tumor cells (41, 42). In fact, Tyr$^{145}$ was identified as one of the major phosphorylation sites. Another relative of moesin is radixin, and it is phosphorylated on tyrosine residues, when NIH3T3 cells, transfected with EGF receptor and radixin, are stimulated with EGF and PDGF (43). These limited data suggest that different cells phosphorylate moesin-like proteins in different ways, but they do not directly address the function of these proteins and the role of phosphorylation during cellular activation.

The phosphorylation site of moesin is located in the sequence KYKTL, which matches the consensus sequence KYXXL motif for actin binding (38). By using F-actin as a probe in an in vitro assay, a 22-amino acid sequence in the carboxy-terminal region of bacterially produced moesin was previously identified as containing the binding site for F-actin (39). This motif is conserved in ezrin, radixin, and EM10, in skeletal and cardiac myosin heavy chains, and in the CapZ β subunit, but not in merlin (Fig. 11 and Refs. 30, 38, 65–68). Although the high degree of sequence homology, centered around the conserved Thr$^{558}$, could be indicative of a common actin-binding and phosphorylation site, and although a synthetic peptide containing this sequence binds actin (68), the KYKXXL motif does not belong to the major actin-binding sites of myosin and is not fully conserved in all myosins. Similar to positively charged binding sites in other actin-binding proteins (69), the sequence in moesin is strongly basic to allow interaction with acidic amino acids in actin. Phosphorylation of threonine within this sequence could conceivably regulate binding interactions between moesin and F-actin by introducing a negative charge.

The following observations, however, point to a more complex relationship. GST-ezrin fusion proteins, lacking a few amino acids from the amino-terminal end, bind to immobilized F-actin in vitro (38), while purified and intact ezrin does not. Neither ezrin nor moesin extracted from neutrophils (presumably in dephosphorylated form) bind directly to F-actin in solution at physiological salt conditions (39). On the other hand, even small peptides from the carboxy-terminal region of moesin bind F-actin in vitro.\(^2\) Furthermore, Aigrain et al. (70) showed that in transiently transfected cells the carboxy-terminal part of ezrin colocalizes predominantly with stress fibers, whereas endogenous ezrin localizes to membrane protrusions (70). Together, these data imply that the carboxy-terminal actin-binding site mentioned above is cryptic in the native protein. On the other hand, there are strong indications that moesin, ezrin, and radixin colocalize with F-actin in filopodial and microvillar membranes in cultured cells and tissues (31–34).

How can these different observations be reconciled? Moesin-like proteins apparently are required for formation or stabilization of filopodial structures, since treatment of cells with moesin, ezrin, and radixin antisense RNA obliterates cell protrusions (77). Colocalization with actin, however, does not necessarily implicate interaction of moesin molecules with actin.

\(^2\) H. Furthmayr, unpublished data.

In fact, cytochalasin treatment of cells depolymerizes F-actin, yet it does not disrupt moesin’s localization to retraction fibers (35). If moesin’s interaction with actin is regulated and reversible, moesin could function as a dynamic link between membrane receptors and treadmilling actin filaments (78). Filopodia are also sites for signaling, and the formation of links between actin filaments and moesin in these structures might very well depend on changes in phosphorylation as a consequence of these signals. According to this idea, phosphorylation would be required to uncover moesin’s actin-binding site by inducing a conformational change. The resting platelet has a discoid shape, but thrombin activation and various other stimuli result in rapid and predictable shape changes, initially characterized by filopodia-like protrusions of the membrane. Moesin phosphorylation could be essential to support the cytoskeletal changes accompanying this process. We have demonstrated that the number of phosphorylated moesin molecules increases for a short time and then declines to a level close to the resting state. This increase in phosphorylation is closely regulated by kinase(s) and phosphatase(s), and their activities, in turn, appear to be influenced by phosphorylation of tyrosine residues. Phosphorylation of moesin molecules is thus the result of a delicate balance of the activity of a presently unidentified kinase and a phosphatase, which are subject to regulation by tyrosine phosphorylation (Fig. 12). The temporary increase in phosphorylation could indicate phosphorylation of one set of molecules and dephosphorylation of another, thus shifting the balance of molecules engaged in binding to actin from one site to another. According to this model, moesin molecules would remain bound to the membrane as opposed to being translocated to other sites. These cytoskeletal events would underlie the plasticity observed in changing from a discoid shape to a star-like shape. Other mechanisms have been proposed to regulate the binding of moesin-like molecules to actin, such as regulation by intermolecular interaction (dimerization), intramolecular interaction between amino-terminal and carboxy-terminal domains (71, 72, 79), similar to the model proposed for vinculin, where head and tail regions interact to modulate binding to talin (74) or interaction with other proteins (73).

The subcellular distributions of moesin molecules in platelets pretreated with kinase or phosphatase inhibitors is markedly different from those of thrombin-activated platelets in the absence of drugs. This could reflect a direct effect on the balance between phosphorylated and dephosphorylated molecules and their actin-binding properties. However, it is more likely that the aberrant cell shapes and motile responses exhibited by drug-treated platelets are the product of effects of these drugs on multiple signal transduction pathways. Because moesin and

![Fig. 12. Regulation of Thr$^{558}$ phosphorylation.](http://www.jbc.org)
Phosphorylation of Platelet Moesin

its family members are both temporally and spatially associated with rapidly moving and changing cell surface structures, especially filopodia and retraction fibers, it is possible that recently discovered GTPases regulate the phosphorylation of moesin.

This pathway has received considerable attention. When platelets are treated with butylinum C3 ADP-ribosyltransferase, inhibition of thrombin-induced aggregation is observed (80). This effect is produced by the specific inhibition of RhoA GTPase, which in cultured fibroblasts modulates the formation of stress fibers and focal adhesion sites (81). Aggregation is a relatively late event in thrombin activation of platelets, but RhoA activity may be more proximal in the GTPase cascade (82). Apparently, these GTPases can be controlled independently by different input signals. Thus, activation of Cdc42 leads to rapid filopodial protrusion exclusively, while the second GTPase in the cascade, Rac, is inhibited (83, 84). Activation of Rac, on the other hand, causes filopodia to extend lamellipodia between preformed filopodia, and, finally, stress fibers and focal contacts are formed due to Rho. The membrane and cytoskeletal changes in fibroblasts to extend lamellopodia between preformed filopo-
Phosphorylation of Threonine 558 in the Carboxyl-terminal Actin-binding Domain of Moesin by Thrombin Activation of Human Platelets
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