

# Moesin, the major ERM protein of lymphocytes and platelets, differs from ezrin in its insensitivity to calpain

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**Abstract** The ERM proteins, ezrin, radixin and moesin, provide regulated linkage of the cytoskeleton with the plasma membrane, particularly in cell surface projections. Ezrin and moesin were found co-expressed, and radixin was not detected, in human blood lymphocytes, monocytes and neutrophils. Moesin is the quantitatively dominant ERM protein in these cells and the only one in platelets. Because  $\text{Ca}^{2+}$  signaling pathways involving calpain cleavages are important in blood cells, we examined ERM protein sensitivity to this protease. A striking difference was discovered: sensitivity of ezrin and resistance of moesin (and radixin) to calpain. In intact stimulated lymphocytes, ezrin was cleaved, while moesin was not, strongly suggesting that differential sensitivity to calpain contributes to specialized functions of these proteins.

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**Key words:** Calpain; Moesin; Ezrin; ERM protein; Platelet; Lymphocyte

## 1. Introduction

Ezrin, radixin, and moesin, the ERM family proteins, localize to, and support, cell surface projections by forming oligomeric head-to-tail structures linking the underlying cytoskeleton with the plasma membrane [1,2]. The proteins are ~75% identical in amino acid sequence. As members (with talin and merlin) of the 4.1 band superfamily, the ERM proteins share that family's structural organization: a globular N-terminal domain, an  $\alpha$ -helical segment and a highly charged C-terminal domain. Their physiological expression patterns differ, strongly suggesting that the proteins are functionally non-identical [3–5], yet the ERM proteins undergo similar interactions when examined as pure proteins and in cell lines and transfected cells [1,2]. The N-terminal domain associates with the plasma membrane and the C-terminal domain with the cytoskeleton [6]. The ERM proteins are essential, for example, for the assembly of actin filaments and focal adhesion complexes mediated by the GTPases Rho and Rac [7]. ERM proteins are found also as dormant monomers in which the N-terminus is associated intramolecularly with the C-terminus [8]. The monomers are subject to complex regulatory mechanisms leading to conformational activation and formation of oligomeric surface linking structures [9].

The focus of our laboratory is blood cells, particularly lymphocytes and platelets, and we are interested therefore in the molecules that regulate their surface projections such as microvilli, filopodia and lamellipodia. Microvilli, the slender

projections that dominate the surface of circulating lymphocytes, function to monitor the blood vessel wall and serve as the locus of adhesion and morphological changes leading to extravasation and activation [10]. The relevance of ERM proteins was indicated by the disappearance of lymphoid cell microvilli on treatment with antisense nucleotides to the three ERM proteins or with single antisense oligonucleotides to moesin [11]. Blood platelets, in contrast to lymphocytes, are smooth-surfaced in the circulating state and, in response to agonists, rapidly develop long filopodia and ruffling lamellae, which contribute to formation of the platelet plug, a crucial event in hemostasis.

As a basis for understanding the role of ERM proteins in lymphocyte and platelet activation events, the present study was undertaken to determine the expression pattern and functional properties of these proteins in blood cells.

## 2. Materials and methods

### 2.1. Reagents

Prostacyclin (PGI<sub>2</sub>), phorbol myristate acetate (PMA), bovine serum albumin, Histopaque 1077 and dextran were from Sigma Chemicals, St. Louis, MO; leupeptin from Boehringer Mannheim Biochemicals, Indianapolis, IN; SDS and NP-40 from BDH Chemicals, Richmond, CA; and ionomycin, calpeptin and  $\mu$ -procalpain (calpain I, porcine erythrocyte, 120 U/mg) from Calbiochem, San Diego, CA.

### 2.2. Antibodies

Monospecific anti-ezrin and anti-moesin rabbit antibodies were affinity-purified as described [12]. Purified anti-radixin antibodies were from rabbits immunized with bacterially expressed GST-radixin. Mouse monoclonal antibodies (mAb) included B27D8, an IgG1 recognizing  $\mu$ -calpain heavy chain [13], and clone 38 anti-moesin amino acids 554–564, an IgG1 from Transduction Labs, Lexington, KY. Affinity-purified goat anti-rabbit and rabbit anti-mouse immunoglobulins were from Cappel, Durham, NC and Pierce Chemicals, Rockford, IL, respectively.

### 2.3. Platelets

Freshly drawn blood from healthy consenting volunteers was collected in acid-citrate-dextrose (ACD; NIH formula A) and centrifuged at  $200\times g$  for 12 min at ambient temperature to separate platelet-rich plasma (PRP). Additional ACD was added (1 part per 3 parts PRP), and the PRP was centrifuged at  $800\times g$  for 15 min. The platelets were resuspended in platelet buffer (10 mM *N*-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid, pH 7.2, 136 mM of NaCl, 2.6 mM of KCl, 0.5 mM of  $\text{NaH}_2\text{PO}_4$ , 2 mM  $\text{MgCl}_2$ , 0.1% glucose, and after addition of ACD (10% of final volume), were pelleted at  $800\times g$  for 10 min.

### 2.4. Lymphocytes and monocytes

The pelleted blood cells (after removal of PRP) were fractionated by Histopaque-1077 density centrifugation as described [14]. The interface lymphocytes and monocytes were harvested, and monocytes depleted from the lymphocytes by adherence. The monocytes were harvested from the tissue culture plates by dislodging with a rubber policeman into cold  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS and washed.

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### 2.5. Neutrophils

Pelleted blood cells were combined 1:1 with 2% dextran in 150 mM NaCl and incubated at  $\sim 22^\circ\text{C}$  for 30 min to sediment erythrocytes. The leukocyte-containing supernatant was aspirated; the cells were collected by pelleting, layered onto Histopaque-1077 and centrifuged at  $800\times g$  for 40 min. The pelleted cells were washed in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS, and residual erythrocytes removed by water lysis.

### 2.6. Erythrocytes

After removal of PRP and the top half of the pelleted blood cells, the remaining packed erythrocytes were suspended in 10 volumes of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS and washed by several cycles of centrifugation for 10 min at 1300 rpm. The erythrocyte preparations contained  $< 1$  leukocyte per  $5\times 10^3$  erythrocytes.

### 2.7. Megakaryocytes

Megakaryocytes were isolated from bone marrow aspirates of volunteer donors [15] by Histopaque-1077 fractionation and culturing to remove adherent material. The non-adherent cells were incubated with anti-GPIIIa (anti-CD61) (mAB Y2/5 from Dako, Carpinteria, CA) and then with rabbit anti-mouse IgG coupled to magnetic beads (Dyna-bead, Lake Success, NY). The megakaryocytes were isolated magnetically and examined by light microscopy.

### 2.8. Endothelial cells

Pooled umbilical vein endothelial cells were grown in primary culture [16] for three passages. At confluence, the cells were quantified by microscopic examination, washed and lysed by adding SDS sample buffer (1% SDS, 62 mM Tris-HCl, pH 6.8, 2% mercaptoethanol, 2 mM EGTA, 25  $\mu\text{g}/\text{ml}$  leupeptin) directly to the plate.

### 2.9. SDS electrophoresis

Lymphocytes, monocytes, neutrophils and HepG2 cells were lysed at  $15\times 10^6/\text{ml}$ , platelets at  $5\times 10^8/\text{ml}$ , erythrocytes at  $7\times 10^8/\text{ml}$ , endothelial cells at  $\sim 6\times 10^6/\text{ml}$ , and megakaryocytes at  $10^5/\text{ml}$  in 1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 50  $\mu\text{g}/\text{ml}$  leupeptin, 2 mM DFP, 2% mercaptoethanol, 20  $\mu\text{g}/\text{ml}$  bromophenol blue, 10% glycerol by first suspending the cells in 0.8 volumes of Tris-NaCl-EGTA-DFP-leupeptin followed by addition of 0.2 volumes of SDS and the remaining reagents and heating for  $> 3$  min at  $100^\circ\text{C}$ . The SDS solubilization protocols for calpain reactions and stimulated lymphocytes are described below. Solubilized samples were fractionated by SDS electrophoresis on 8% acrylamide gels (Novex, San Diego, CA) using pH 8.3 running buffer [17].

### 2.10. Immunoblotting

Polypeptides were transferred to nitrocellulose at constant 80 mA at  $\sim 22^\circ\text{C}$  for 16 h. The membrane was blocked with 2% normal goat serum in PBS/0.05% Tween-20 for 20 min, washed and incubated for 2 h with 20 ng/ml rabbit antibodies to moesin or ezrin or radixin. The membranes were washed and incubated with  $^{125}\text{I}$ -labeled goat anti-rabbit IgG (0.5–1.0  $\mu\text{g}/\text{ml}$ ) for 1 h, washed and exposed to Phosphor-screen, and bands were quantified using Phosphorimager Storm 860 and Image Quant v1.1 program (Molecular Dynamics, Sunnyvale, CA). Alternatively, membranes blocked with 2% normal rabbit serum were stained with B27D8 anti-calpain (ascites at 1:20 000) or clone 38 anti-moesin (125 ng/ml) and detected with  $^{125}\text{I}$ -labeled rabbit anti-mouse IgG.

### 2.11. Quantitation of ezrin and moesin

Lysates of blood cells were examined by immunoblot in parallel with dilutions of pure ezrin isolated from human placenta [18], which served as an internal standard curve. Moesin was similarly quantified using a standard curve of full-length moesin, which was expressed in and purified from *Escherichia coli* using procedures identical to those for full-length ezrin [19].

### 2.12. Calpain reactions in broken cell preparations

Platelets at  $10^9/\text{ml}$  in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS containing 1 mM of EGTA and 2 mM mercaptoethanol were lysed by sonication [14], clarified by centrifugation and stored in aliquots at  $-80^\circ\text{C}$ . Lymphocytes ( $15\times 10^6/\text{ml}$ ) and HeLa cells ( $25\times 10^6/\text{ml}$ ) were lysed in 0.5% NP-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM DFP, 25  $\mu\text{g}/\text{ml}$  leupeptin, 2 mM EGTA and clarified by centrifugation.

Platelet sonicates (or platelet sonicates combined 1:1 with lymphocyte lysate) were preincubated at  $\sim 22^\circ\text{C}$  for 3 min;  $\text{CaCl}_2$  was added to 6 mM and incubation continued at  $\sim 22^\circ\text{C}$  for 0–30 min. The reactions were stopped with an equal volume of  $2\times$  SDS sample buffer and heating at  $100^\circ\text{C}$  for 2 min. HeLa cells lysates were combined 1:1 with  $\mu$ -procaspain (calpain I from porcine erythrocytes) in 20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.2 mM mercaptoethanol, and after addition of  $\text{CaCl}_2$  (6 mM), were incubated at  $\sim 22^\circ\text{C}$  for 0–20 min, and processed as above. Pure ezrin or pure moesin at 40  $\mu\text{g}/\text{ml}$  was treated with procaspain and  $\text{Ca}^{2+}$  as described for HeLa cell lysates.

### 2.13. Lymphocyte stimulation

Lymphocytes at  $1.5\times 10^7/\text{ml}$  in RPMI 1640 medium with 5% fetal calf serum, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin were precultured for  $\sim 10$  min at  $37^\circ\text{C}$  prior to addition of PMA

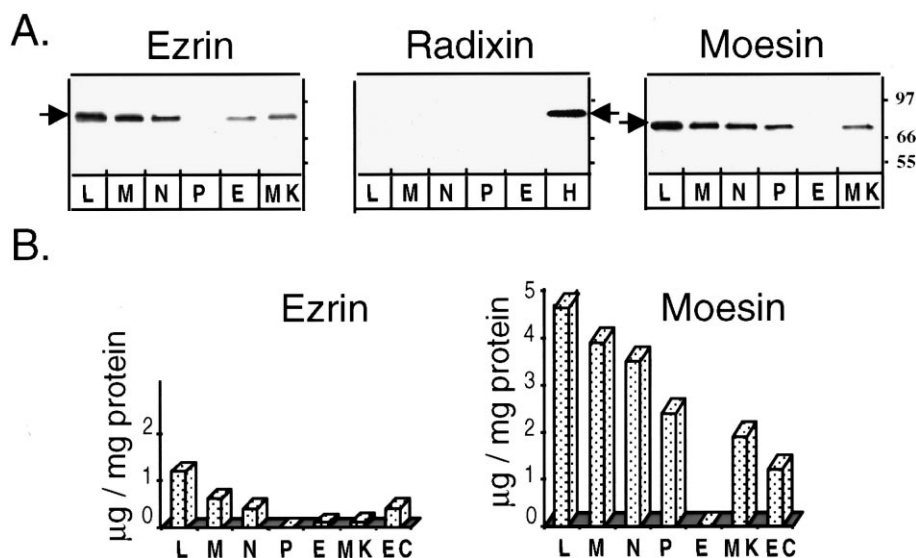


Fig. 1. Human hematopoietic cells and control cells examined for expression of ezrin, radixin and moesin. A: Immunoblots. Similar total protein levels were stained for ezrin (left panel), radixin (center panel) and moesin (right panel). Shown are L, lymphocytes; M, monocytes; N, neutrophils; P, platelets; E, erythrocytes; MK, bone marrow megakaryocytes and, as a control for radixin, H, HepG2 cells. Arrows indicate ezrin and radixin migrating at 81 kDa and moesin at 77 kDa. B: Quantitation of ezrin and moesin in isolated cells (details in Section 2). Levels of ezrin (left) and moesin (right) are expressed as  $\mu\text{g}/\text{mg}$  of total cell protein. 'EC' indicates endothelial cells. Values are the mean of  $\geq 3$  assays of 2–3 lysates; S.E.M. was  $< 5\%$ .

(10 ng/ml) and ionomycin (1  $\mu$ M). After 0–30 min at 37°C, the cells were lysed by adding 5 $\times$ SDS sample buffer and heating at 100°C. To inhibit calpain action, the lymphocytes were preincubated with 50  $\mu$ g/ml calpeptin for 20 min at 37°C prior to addition of PMA and ionomycin.

#### 2.14. Protein quantitation

Protein in cell lysates was quantified with Nano-Orange (N-6666 kits; Molecular Probes, Eugene, OR) using bovine albumin as standard. Fluorescence was measured with excitation at 485 nm and emission at 590 nm.

### 3. Results

#### 3.1. Ezrin and moesin expression in blood cells

To determine the expression pattern of ERM proteins, immunoblots of isolated human blood cells were stained with rabbit antibodies specific for each protein. On analysis of similar amounts of total cell protein, ezrin was found at readily detectable levels in lymphocytes, monocytes and neutrophils and at low levels in erythrocytes (Fig. 1A, left). Although ezrin was readily detected also in megakaryocytes,

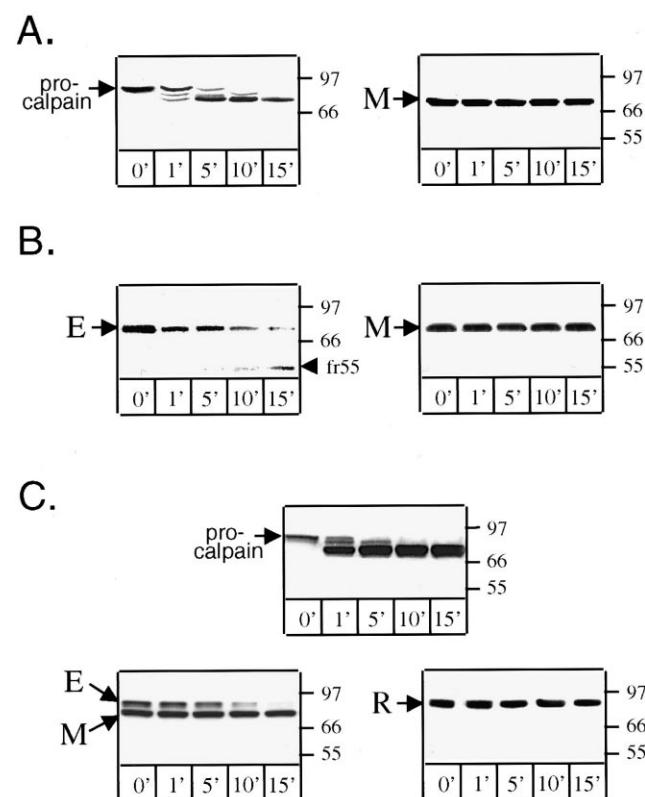


Fig. 2. Sensitivity of ezrin, moesin and radixin to calpain. Broken cell preparations were incubated at  $\sim 22^\circ\text{C}$  for the indicated time and analyzed by immunoblots. A: Platelet sonicates incubated with  $Ca^{2+}$ . The left panel shows conversion of endogenous 80 kDa pro-calpain (pro-calp) to active 76 kDa calpain (arrow); the right panel shows moesin (M) unaltered after 15 min of calpain exposure. B: Combined lymphocyte and platelet lysates incubated with  $Ca^{2+}$ . The left panel shows time-dependent cleavage of ezrin (E) and formation of the short-lived 55 kDa fragment (fr-55). The right panel shows unaltered moesin (M) in the same reaction. C: HeLa cell lysate incubated with pure pro-calpain and  $Ca^{2+}$ . The upper panel shows calpain activation. The lower left panel, a blot stained with rabbit antibodies that detect both ezrin and moesin, shows time-dependent decrease of ezrin and unaltered moesin. The lower right panel shows unaltered radixin (R) detected with specific antibodies.

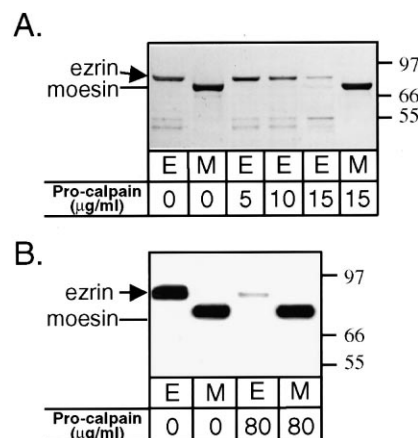


Fig. 3. Sensitivity of pure ezrin and moesin to calpain. Pure ezrin and moesin were separately incubated with pure pro-calpain and  $Ca^{2+}$  at  $\sim 22^\circ\text{C}$  for 20 min. A: Coomassie blue-stained gel shows ezrin (E) incubated with 0, 5, 10 and 15  $\mu$ g/ml pro-calpain and moesin (M) incubated with 0 and 15  $\mu$ g/ml pro-calpain. B: Immunoblot stained with a mAb that recognizes a shared epitope shows ezrin (E) and moesin (M) incubated with 0 and 80  $\mu$ g/ml pro-calpain.

the platelet precursor cells in bone marrow, it was not found in platelets (Fig. 1A) in verification of a previous report [20]. The apparent lack of ezrin in platelets is unlikely to be due to proteolysis because (i) the lysates were prepared with protease inhibitors and (ii) SDS gels stained with Coomassie blue showed no detectable degradation of platelet proteins (data not shown). On staining with radixin-specific antibodies, no reactive protein was detected in lymphocytes, monocytes, neutrophils, platelets or erythrocytes, but was detected in HepG2 cells [21] used as positive control (Fig. 1A, center). Immunoblots with moesin-specific antibodies detected moesin in lymphocytes, monocytes, neutrophils and also in platelets and megakaryocytes (Fig. 1A, right).

#### 3.2. Levels of ezrin and moesin in blood cells

Because the intensity of immunoblot signals depends only partially on antigen density, we quantified ezrin and moesin in blood cells by comparing their immunoblot signals to those of known amounts of pure ezrin or moesin. Ezrin levels were found to vary from a low of  $<0.1$   $\mu$ g/mg cell protein in erythrocytes and megakaryocytes to 0.5, 0.7 and 1.3  $\mu$ g/mg protein, respectively, in neutrophils, monocytes and lymphocytes (Fig. 1B, left).

Surprisingly, moesin levels in blood cells were found to be very high. Whereas endothelial cells, which were previously noted to have a high moesin content [5,4], contain 1.5  $\mu$ g moesin/mg cell protein, megakaryocytes have 1.8  $\mu$ g; platelets, 2.5  $\mu$ g; neutrophils, 3.0  $\mu$ g; monocytes, 4.0  $\mu$ g; and lymphocytes, the highest level, 4.8  $\mu$ g/mg cell protein (Fig. 1B, right). Cumulatively, these findings identify moesin as the quantitatively dominant ERM protein in white blood cells and, as previously reported, the sole ERM protein in platelets.

#### 3.3. Ezrin and moesin differ in sensitivity to calpain in vitro

In this section, we compared moesin and ezrin for susceptibility to proteolysis, a putative *in vitro* correlate of termination of ERM function (see Section 4). The neutral cysteine protease calpain was examined because it is a known effector of  $Ca^{2+}$ -mediated platelet and lymphocyte activation path-

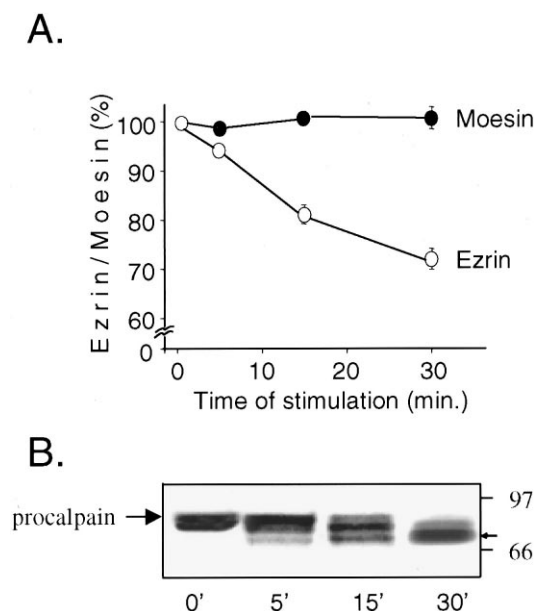


Fig. 4. Cleavage of ezrin and persistence of moesin in blood lymphocytes stimulated with PMA plus ionomycin. Lymphocytes in serum-containing medium were cultured with PMA and ionomycin. At the indicated times, the cells were lysed, and ezrin, moesin and calpain were examined by immunoblotting. A: Quantitation of ezrin and moesin in treated cells expressed as percent relative to starting cells. Data are the mean  $\pm$  S.E.M. of four experiments with lymphocytes from different donors. B: Immunoblot from a representative experiment demonstrating activation of endogenous lymphocyte calpain.

ways [22,23]. Calpain, an inactive proenzyme in resting cells, is autoproteolytically activated on exposure to elevated  $\text{Ca}^{2+}$ . Previous studies showed that ezrin is cleaved by calpain [24,25] and the related 4.1 superfamily proteins talin [22] and merlin [26] are also among the select calpain substrates; moesin had not been examined.

To examine moesin's sensitivity to calpain, we generated platelet sonicates as an approximation of the intraplatelet environment and as a rich source of procalpain [27]. Addition of  $\text{Ca}^{2+}$  converted procalpain to active calpain, which was verified by altered SDS mobility of the calpain heavy chain (Fig. 2A, left). Coomassie blue-stained SDS gels demonstrated cleavage of the endogenous calpain substrates actin binding protein and talin [22] (data not shown). However, no degradation of moesin was observed up to 15 min (Fig. 2A, right).

To verify moesin's insensitivity to calpain by simultaneously examining ezrin, lymphocyte lysates were combined with platelet sonicates prior to activation of endogenous calpain. Time-dependent degradation of ezrin began 1 min after  $\text{Ca}^{2+}$  addition, producing the previously described [24,25] short-lived 55 kDa fragment (fr-55, Fig. 2B, left panel); ezrin degradation was not observed when  $\text{Ca}^{2+}$  was omitted or the calpain inhibitor leupeptin added (data not shown). No degradation of moesin was observed up to 15 min (Fig. 2B, right).

Since the amounts of moesin and ezrin are unequal in the combined lymphocyte/platelet lysates, we also examined lysates of HeLa epithelioid cells, which contain comparable amounts of ezrin and moesin. Pure  $\mu$ -procalpain (from porcine erythrocytes) was added and activated by addition of  $\text{Ca}^{2+}$ . Immunoblots showed time-dependent activation of cal-

pain (Fig. 2C, upper panel). Ezrin underwent time-dependent cleavage, and moesin in the same samples remained unaltered (Fig. 2C, lower left panel). Since HeLa cells, unlike blood cells, also express radixin, aliquots of the treated HeLa cell lysates were also immunoblotted for radixin, which, like moesin and unlike ezrin, was found unaltered after 15 min exposure to calpain (Fig. 2C, lower right panel).

Finally, pure ezrin and moesin were separately incubated with pure  $\mu$ -procalpain in the presence of  $\text{Ca}^{2+}$ . Coomassie blue-stained gels revealed dose-dependent cleavage of ezrin by 10 and 15  $\mu\text{g/ml}$  calpain (Fig. 3A). The resistance of pure moesin to 15  $\mu\text{g/ml}$  calpain was demonstrated by Coomassie blue-stained gels (Fig. 3A, last lane), and its resistance to 80  $\mu\text{g/ml}$  calpain was demonstrated by immunoblot (Fig. 3B). These cumulative findings verify the calpain sensitivity of ezrin by showing its efficient and complete cleavage and demonstrate that moesin (and also radixin) remains resistant even to several-fold higher calpain levels.

### 3.4. Differential fate of ezrin and moesin in intact cells

To examine the calpain sensitivity of ezrin and moesin in intact blood cells, we focused on lymphocytes, which express both ezrin and moesin and which respond to proliferation-inducing stimuli by a  $\text{Ca}^{2+}$  signaling pathway that includes calpain activation [23]. Isolated lymphocytes were stimulated by the combination of PMA, a protein kinase C activator, and ionomycin, a  $\text{Ca}^{2+}$  ionophore; this well-characterized T-cell stimulus bypasses the T-cell receptor/CD3 complex and induces lymphocyte proliferation after  $\sim 48$  h [28].

On culture with PMA and ionophore, a time-dependent decrease was detected in the lymphocyte content of intact ezrin (Fig. 4A); the change amounted to a  $29 \pm 5\%$  decrease after 30 min ( $n = 4$ ) (Fig. 4A). In contrast, moesin was quantitatively recovered in the same cell lysates (Fig. 4A). Activation of endogenous lymphocyte calpain was verified (Fig. 4B). When lymphocytes were pretreated with calpeptin, a cell-permeating calpain inhibitor [29], ezrin levels did not decrease. After 30 min, the levels of intact ezrin remaining in calpeptin-pretreated lymphocytes were  $102 \pm 4.5\%$  and  $100 \pm 3.8\%$  ( $n = 3$ ), respectively, in cultures with and without PMA and ionomycin, strongly suggesting that the decrease of ezrin induced by PMA and ionomycin is calpain-dependent. These findings demonstrate that ezrin is cleaved in intact activated lymphocytes in a calpain-dependent reaction and moesin remains unaltered.

## 4. Discussion

Immunoblots with specific antibodies detected ezrin and moesin in white blood cells: lymphocytes, monocytes and neutrophils, and also in megakaryocytes, platelet precursor cells from bone marrow. Previous studies have shown that the only ERM protein present in platelets is moesin [20], and we have now quantified the level of moesin in all these cells. Indeed, moesin expression levels in blood cells are high by any standard, amounting in lymphocytes to  $\sim 0.5\%$  of total cell protein. Radixin was not found in any blood cell examined.

As a step toward understanding their function, we compared the blood cell ERM proteins ezrin and moesin for sensitivity to the protease calpain, an important effector of  $\text{Ca}^{2+}$  signaling and activation-associated morphological changes in

blood cells. Several *in vitro* approaches were used to examine calpain sensitivity: platelet sonicates studied with endogenous calpain to preserve the physiological environment, HeLa cell lysates studied with exogenous calpain, which allowed comparable levels of ezrin and moesin to be examined, and also pure moesin and ezrin treated with pure calpain. In all systems, a dramatic difference was found in that ezrin was readily and completely proteolysed, and moesin was insensitive even at several-fold higher calpain levels. An advantage of the HeLa cell system is that endogenous radixin, which was not found in blood cells, could also be examined; radixin was also resistant to calpain. Different sensitivity to calpain is one of the few divergent biochemical reactions of ezrin and moesin, and we propose that this feature provides a basis for non-redundant functions of these proteins.

The ERM proteins have different expression patterns and levels, as shown here for blood cells, strongly suggesting that each has specialized or unique functions, yet the three proteins are structurally similar and have been considered functionally equivalent. These findings imply that the basis for specialized/unique functions will be found at the level of regulatory events, i.e. either selective recruitment of individual proteins to form functional linker structures and/or selective termination of linker structures involving individual proteins.

Selective recruitment was suggested by the finding that, in cells expressing the three ERM proteins, each exhibited a distinct receptor-specific phosphorylation pattern, and thus individual proteins might be recruited and functionally activated in response to different receptor signals [30]. Less obvious is the interpretation of EGF-induced recruitment of ezrin and moesin to transient membrane ruffles in that both proteins are recruited, but only ezrin is tyrosine-phosphorylated [18,12].

The hypothesis that different sensitivity to calpain provides a basis for selective termination of ezrin's and moesin's specialized linker structures is supported by theoretical considerations and experimental evidence. Since the ability of ezrin and moesin to function as linker proteins requires sites in the N-terminus that bind plasma membrane proteins and sites in the C-terminus that bind the actin cytoskeleton [6], proteolytic cleavage has the capacity to disrupt membrane-cytoskeleton linkages. Evidence strongly suggesting that calpain sensitivity suffices as a basis for terminating ezrin's linker function is provided by studies in gastric parietal cells in which ezrin cleavage by calpain was linked to inhibition of proton secretion [24]. In endothelial cells, ezrin cleavage by calpain was associated with stimulation of locomotion and abolition of ezrin's ability to associate with  $\beta$ -actin [25].

A related pathological example of calpain sensitivity serving as the basis for termination of ERM-like function is provided by merlin, a 4.1 band superfamily protein closely related to the ERM proteins, sharing ~65% amino acid identity over its N-terminal domain [31]. Merlin functions as a tumor suppressor. Tumors (schwannomas and meningiomas) arise in most cases due to mutations of the merlin gene leading to non-expression of merlin protein, but in other cases are due to stable overactivation of the calpain system, which causes depletion of merlin by proteolysis [26].

The present study demonstrated that ezrin and moesin of blood lymphocytes exhibit a divergent response to signaling involving  $\text{Ca}^{2+}$  transients induced by PMA and  $\text{Ca}^{2+}$  ionophore. Whereas ezrin was cleaved, moesin remained intact.

Similar results were obtained when lymphocytes were activated with the more physiological stimulus anti-CD3 mAb (Shcherbina et al., in preparation). These findings suggest that moesin, but not ezrin, retains the ability to function during and subsequent to  $\text{Ca}^{2+}$  signaling. Indeed, ezrin and moesin may function sequentially to support different morphological structures required as lymphocytes differentiate, i.e. early acting ezrin in resting cells and moesin contributing to surface projections in lymphocytes that have undergone  $\text{Ca}^{2+}$  signaling and activation. Circulating lymphocytes are spheroidal cells with a surface dominated by slender microvilli that serve to monitor the vascular wall [10]. As part of the early response to proliferation-inducing stimuli, lymphocytes undergo cytoskeletal remodeling and become polarized [32–34]. In resting lymphocytes, ezrin and moesin were found by fluorescent microscopy to co-localize in surface microvilli, and these structures were rapidly disassembled on cell activation (Shcherbina et al., in preparation). Since ezrin and moesin can form heterodimers *in vivo* [35], early acting linker structures in resting lymphocytes may be composed of both ezrin and moesin, yet be disassembled upon lymphocyte activation by calpain proteolysis of only ezrin. In support of this theory, fluorescent microscopy revealed weak ezrin staining homogeneously distributed in the cytosol, and moesin found as the sole ERM protein in the uropods of polarized activated T-lymphoblasts [36].

In summary, ezrin and the predominant moesin represent the ERM proteins in blood cells. Cleavage of ezrin by calpain and insensitivity of moesin to this protease are documented as biochemically distinguishing features. Calpain-dependent cleavage of ezrin and persistence of moesin is demonstrated in the early response of intact lymphocytes to proliferation-inducing stimulation. Together, these findings suggest that different susceptibility to calpain contributes to the specialized non-redundant functions of ezrin and moesin.

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