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Moesin, Ezrin, and p205 Are Actin-binding Proteins Associated with Neutrophil Plasma Membranes

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Actin-binding proteins in bovine neutrophil plasma membranes were identified using blot overlays with 125I-labeled F-actin. Along with surface-biotinylated proteins, membranes were enriched in major actin-binding polypeptides of 78, 81, and 205 kDa. Binding was specific for F-actin because G-actin did not bind. Further, unlabeled F-actin blocked the binding of 125I-labeled F-actin whereas other acidic biopolymers were relatively ineffective. Binding also was specifically inhibited by myosin subfragment 1, but not by CapZ or plasma gelsolin, suggesting that the membrane proteins, like myosin, bind along the sides of the actin filaments. The 78- and 81-kDa polypeptides were identified as moesin and ezrin, respectively, by co-migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoprecipitation with antibodies specific for moesin and ezrin. Although not present in detectable amounts in bovine neutrophils, radixin (a third and closely related member of this gene family) also bound 125I-labeled F-actin on blot overlays. Experiments with full-length and truncated bacterial fusion proteins localized the actin-binding site in moesin to the extreme carboxy terminus, a highly conserved sequence. Immunofluorescence micrographs of permeabilized cells and cell “footprints” showed moesin co-localization with actin at the cytoplasmic surface of the plasma membrane, consistent with a role as a membrane-actin–linking protein.

INTRODUCTION

Chemotaxis, adhesion, and cell shape changes are biologically important events during development, in inflammatory and wound healing responses, and in the invasive phase of tumor progression (Kiehart, 1990; Rao et al., 1991; Herman, 1993; Tsukita et al., 1993; Klymkowsky et al., 1994). The neutrophil is a well-studied model system for these phenomena and an interesting biological system in its own right for mechanistic studies of invasion, cell motility, and host defense against foreign material (Baggiolini et al., 1993; Nauseef, 1993). The cellular response to chemoattract factors is mediated through transmembrane receptors, recruitment or activation of adhesion receptors at the cell surface, and reorganization of the underlying actin-based cytoskeleton (Stossel, 1988; Springer, 1990). Conversely, the actin-based membrane skeleton plays a role in modulating receptor affinity and accessibility (Jesaitis et al., 1984; Särndahl et al., 1989).

Although very little is known about membrane-associated cytoskeletal proteins in neutrophils, filamentous meshworks are associated with the cytoplasmic surfaces of adherent neutrophils after mechanical disruption (Boyles et al., 1979; Boyles et al., 1981) or selective solubilization with the nonionic detergent, Triton X-100 (Sheterline et al., 1981). A membrane fraction enriched in neutrophil plasma membranes contains actin and the actin-binding membrane skeletal proteins, fodrin (nonerythrocyte spectrin), protein 4.1, and ponticulin (Stevenson et al., 1989; Wuestehube et al., 1989). Although the chemotactic receptor for f-Met-Leu-Phe associates with actin, directly or indirectly

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(Jesaitis et al., 1993), and the integrin β2-subunit (CD18) binds the actin-bundling protein α-actinin (Pavalko et al., 1993), the significance of these interactions for neutrophil function is unclear.

Because of the transient nature of cell surface protrusions, relatively little is known about linkages between the plasma membrane and the actin-based cytoskeleton in dynamic cells, such as neutrophils (reviewed by Bourguignon, 1992; Luna and Hitt, 1992; Bretscher, 1993; Hitt and Luna, 1994). Among the proteins that co-localize with actin at cell surface extensions are ezrin (also called cytovillin), moesin, and radixin, a group of closely related proteins that contain structural similarities to erythrocyte protein 4.1 (Bretscher, 1983; Bretscher, 1993; Goslin et al., 1989; Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Tsukita et al., 1993; Arpin et al., 1994). These proteins are usually membrane-associated cytoplasmic constituents, but binding of moesin and ezrin to extracellular cell surfaces has been observed (Lankes et al., 1988; Fazioli et al., 1993). The involvement of moesin, ezrin, and radixin in protrusive activity is suggested by the observation that microvilli, filopodia, and ruffles disappear from the surfaces of thymoma cells treated with antisense oligonucleotides against all three proteins (Takeuchi et al., 1994). In addition, these antisense oligonucleotides disrupt cell-cell and cell-substrate adhesion, a finding in agreement with the observation that some (Sato et al., 1992), but not all (Arnieva et al., 1994), antibodies against radixin stain cell-cell adherens junctions and focal contacts.

Until recently, the evidence that ezrin, moesin, and radixin associate with the cytoskeleton has been indirect. First, preparations highly enriched in radixin have been reported to block the barbed ends of actin filaments (Tsukita et al., 1989), but direct binding of radixin to actin has not been demonstrated and interactions of purified ezrin or moesin with actin have not been detected under physiological conditions (Krieg and Hunter, 1992; Bretscher, 1993; Fazioli et al., 1993). Second, an epitope-tagged construct consisting of the carboxy-terminal half of ezrin (amino acids 280–585) has been shown to localize to stress fibers and other cortical actin structures (Algrain et al., 1993), suggesting that sequences in this region of the protein mediate interactions with the actin-based cytoskeleton. While the current study was in progress, this suggestion was confirmed by the demonstration that glutathione-S-transferase (GST)-fusion proteins containing as few as 34 amino acids from the carboxy terminus of ezrin bind to filamentous, but not to monomeric, actin (Turunen et al., 1994). Because this sequence is nearly identical with the carboxy termini of moesin and radixin (Lankes et al., 1993), the expectation is that all three proteins can bind to F-actin through their conserved carboxy termini.

In the present report, we use blot overlays with 125I-labeled F-actin (Chia et al., 1991) to identify actin-binding proteins in a plasma membrane–enriched fraction purified from bovine neutrophils. Three membrane polypeptides, with apparent molecular masses of 78, 81, and 205 kDa, bind specifically to F-actin by this technique. Because moesin fragments block the binding of F-actin to all three proteins, binding apparently involves the sides, rather than the barbed ends, of the actin filaments. The 78-kDa and 81-kDa proteins are identified as moesin and ezrin, respectively, using antibodies specific for each. Moesin co-localizes with F-actin at the cytoplasmic surface of the plasma membrane, supporting a role for this protein in the organization of the neutrophil membrane skeleton. Finally, F-actin is shown to bind purified, bacterially expressed moesin and radixin. In agreement with previous results (Turunen et al., 1994), the actin-binding site detected by blot overlays is located at the conserved carboxy terminus because truncation of 22 amino acids from this end of moesin results in complete loss of actin binding. These observations suggest that moesin, ezrin, and p205 are all actin-binding proteins associated with the neutrophil membrane skeleton.

**MATERIALS AND METHODS**

**Neutrophil Plasma Membranes**

After hypotonic lysis of erythrocytes, bovine blood neutrophils were isolated by centrifugation through pre-formed gradients of isotonic Percoll (Pharmacia, Uppsala, Sweden) and purified as described by Bourguignon et al. (1983). The procedure described by Mottola et al. (1980) was followed, except that Hanks’ deficient salt solution (HDSS)1 was used instead of Krebs–Ringer solution. HDSS consisted of 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH2PO4, 6.3 mM NaHPO4, 5.55 mM glucose, 4.17 mM NaHCO3, 10 mM HEPES, pH 7.4.

A cell fraction enriched in plasma membranes, called the γ fraction, was prepared by nitrogen cavitation and density gradient centrifugation through Percoll (Del Buono et al., 1989). The published procedure was followed, except that cells pretreated with 5 mM diisopropylfluorophosphate (Sigma Chemical, St. Louis, MO) were suspended at densities of 1–5 × 109 cells/ml in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1 mM ATP, 1.25 mM EGTA, 10 mM PIPES, pH 7.3) containing 10 μM leupeptin (Sigma Chemical), 5 μM pepstatin (Sigma Chemical), 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical), and 30 μM N-tosyl-L-phenylalanine chloromethyl ketone (Sigma Chemical); the cells were cavitated at 400 psi for 20 min on ice. The cavitate was centrifuged at 500 × g for 10 min, and the supernatant was spun through a cushion of Percoll, which had been adjusted to a density of 1.080 with relaxation buffer, at 46,000 × g for 35 min. The cytosol, plasma membrane fraction, and granule fraction were harvested and recentrifuged at 140,000 × g for 2 h. Note that by using a Percoll cushion instead of a gradient, we recovered the azurophilic (α), specific (β), and large granules (Borregaard et al., 1983; Gennaro et

1 Abbreviations used: HDSS: Hanks’ deficient salt solution consisting of 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH2PO4, 0.63 mM NaHPO4, 5.55 mM glucose, 4.17 mM NaHCO3, 10 mM HEPES, pH 7.4; PIPES buffer, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl2, pH 6.9; TBS; 90 mM NaCl, 0.5% (vol/vol) Tween-20, 10 mM Tris-HCl, pH 7.5.
al., 1983; Del Buono et al., 1989) in a single fraction. Fractions were stored separately at −80°C.

**Cell Surface Biotinylation**

Washed neutrophils were suspended in 5 mM sulfo-NHS biotin (Pierce Chemical, Rockford, IL). HDSS at 10^5 cells/mL and mixed gently at 4°C for 10 min. The reaction was quenched by adding glycine, pH 7.4, to a final concentration of 50 mM, the cells were washed once with HDSS, and the y fraction was washed with 1% Tween-20, using bovine serum albumin as the standard. Varied amounts (−0.5–80 μg) of cells and membranes were run on SDS-polyacrylamide gels (Laemmli, 1970) and electrotransferred onto nitrocellulose (Towbin et al., 1979). After blocking with 3% BSA in TBST (90 mM NaCl, 0.5% (vol/vol) Tween-20, 10 mM Tris-HCl, pH 7.5), the blots were probed with 125I-labeled streptavidin (Goodloe & Luna, 1987). The amount of bound streptavidin was quantified with a PhosphorImager and ImageQuant v3.2 software (Molecular Dynamics, Sunnyvale, CA).

**Antibodies**

Rabbit polyclonal anti-ezrin pASε (previously called 90–3), prepared against human ezrin, recognizes only ezrin on immunoblots but sediments some moesin in immunoprecipitations (Lankes and Furthmayr, 1991). Rabbit polyclonal anti-radixin pASβ (Amieva et al., 1994), prepared against recombinant human radixin, is specific for radixin both in immunoblotting and immunoprecipitations (see below). Rabbit polyclonal anti-moesin pASMoER (previously called 90–7), generated against calf moesin, recognizes all three proteins in this gene family by both analytical techniques (Lankes and Furthmayr, 1991). However, after adsorption with recombinant human ezrin and radixin, and affinity purification of the antiserum with recombinant human moesin, affinity-purified antibody pAbMo is specific for moesin both by immunoblotting and by immunoprecipitation (Amieva and Furthmayr, unpublished observations). Monoclonal anti-moesin mAb38/87, prepared against calf moesin, recognizes both radixin and moesin on immunoblots (see below), reacts poorly in immunoprecipitations, but reacts with moesin by immunofluorescence staining of cells and tissues (Lankes et al., 1988).

**Fluorescence Microscopy**

**Cell Footprints.** Bovine neutrophils were isolated as described above, counted, and resuspended to 1–5 x 10^6 cells/mL with HDSS. Portions (50 μL) of the cell suspension were incubated for 10 min at 21–22°C on coverslips cleaned in 1 N HCl overnight and then rinsed five times in distilled water. The coverslips were flooded with 138 mM NaCl, 2.7 mM KCl, 0.6 mM CaCl2, 1.0 mM MgCl2, 10 mM phosphate, pH 7.4, daily made to 10 mM glucose before using, and the incubation was continued for an additional 60 min. The buffer was replaced with 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl2, pH 6.9 (PHEM buffer; Schliva and Van Blerkom, 1981; Hartwig et al., 1989), a second coverslip was placed over the first, and the two were lightly pressed together for 10 s. The top coverslip was removed and placed into a petri dish, sample side up. Adherent cell fragments (cell footprints) were fixed for 15 min at 21–22°C with 0.75 ml of lipid dye/fix solution [PHEN buffer containing 1% formaldehyde and 0.25 mg/ml 5-[N-tetradecanoyl]aminofluorescein (Molecular Probes, Eugene, OR)]. After removal of the fixation solution, the adherent cell fragments were stained for 30 min at 21–22°C with 0.5 ml of rhodamine-phalloidin solution [PHEM buffer containing 100 nM rhodamine-phalloidin (Molecular Probes)], rinsed three times with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.2), and air dried. Each sample was mounted with 10 μl of 50% glycerol in PBS, sealed with Cherry Burst nail enamel (Cutex, Greenwich, CT), and observed in an Axioskop (Carl Zeiss, Thornwood, NY).

To stain cell footprints for moesin, the same procedure was followed through the cell cleavage step. Cell fragments were fixed for 15 min with 1% formaldehyde in PHEM buffer and stained for 30 min in 0.5 ml PHEM buffer containing 6 μl/ml of fluorescein-phallloidin (Molecular Probes). After rinsing with PBS, the cell fragments were blocked for 1 h with 1 ml 10% horse serum in PBS, washed three times with PBS, and incubated for 1 h with 50 μl of anti-moesin mAb38/87 (1/100 in blocking solution). After three 5-min washes in PBS, 10% horse serum, each sample was incubated for 1 h with 50 μl of a 1/100 diluted Texas Red-conjugated affinity-purified rabbit anti-mouse IgG (Cappel/Or ganon Teknika, Durham, NC). Coverslips were washed and mounted as described above.

**Immunofluorescence.** Neutrophils were isolated and allowed to attach to acid-cleaved coverslips for 1 h, as described above. Adherent cells were washed three times with HDSS, 0.1% BSA and fixed for 20 min in 1% paraformaldehyde, 25 mM sodium 2-N-morpholinoethanesulfonate (MES), pH 7.5. After three washes in PBS, half of the samples were permeabilized for 1 min with 1% Triton X-100 (Sigma Chemical) in PBS and rinsed with PBS. The other half of the samples remained in PBS without Triton. All of the samples were blocked overnight at 4°C with 10% horse serum in PBS. Incubations with anti-moesin mAb38/87 and secondary antibody were the same as described for cell footprints, except that the wash between incubations was in 10% horse serum. Control samples of both permeabilized and unpermeabilized cells were prepared with no primary antibody. The coverslips were washed and mounted as for cell footprints.

**F-Actin Blot Overlays**

Cells and cell fractions were denatured at 70°C for 10 min in Laemmli denaturing buffer (Laemmli, 1970), and several amounts of each fraction were electrophoresed into discontinuous 1.5-mm SDS gels at 30 mA for ~5 h at 21–22°C. After removal of surface SDS by a brief wash with transfer buffer, proteins were electrotransferred to nitrocellulose (0.45-μm pore size; Schleicher & Schuell, Keene, NH) at 6 V/cm for 16–20 h at 4°C (Towbin et al., 1979) using a Transblot Cell (Bio-Rad Laboratories, Hercules, CA). Nitrocellulose blots were blocked in 5% milk, TBST for either 1 h at 21–22°C or overnight at 4°C. Apparent molecular masses (Mr) were determined by comparison with prestained standards (Amersham Corporation, Arlington Heights, IL). The bottom edges of these standards are assumed to approximate the Mr of the unlabeled proteins.

Cell footprints purified G-actin was labeled with 125I-Bolton Hunter reagent (Dupont NEN, Wilmington, DE) and stored in dialysis (Schwarz and Luna, 1986; Wuestehube and Luna, 1987). For blot overlays with F-actin, 125I-labeled actin was polymerized in the presence of rabbit plasma gelsolin, stabilized with phallolidin, and stored at 1 mg/ml on ice (Chia et al., 1991). Unless stated otherwise, phallolidinstabilized 125I-labeled F-actin was diluted to final concentrations of 50 μg/ml with 5% (wt/vol) nonfat powdered milk (Carnation Co., Los Angeles, CA), 5 μM phallolidin (Boehringer Mannheim, Indianapolis, IN) in TBST before incubation with nitrocellulose blots for 2 h at −21°C (Chia et al., 1991). Blot overlays with 125I-labeled G-actin were performed similarly with freshly prepared solutions, except that 0.2 mM ATP was added to stabilize the actin. As found previously (Chia et al., 1991), controls showed that the effects of phallolidin and ATP were limited to their roles in stabilizing actin polymer and monomer, respectively (our unpublished observations).

**Competition with Unlabeled Proteins**

**Proteins.** Polyspecific antibody (15–50 kDa) and heparin (sodium salt, Grifols) were purchased from Sigma Chemical, Inc. Affinity-purified tubulin (Williams and Lee, 1982), a generous gift from Dr. Curtis Willkerson (Worcester Foundation for Experimental Biology, Shrewsbury, MA), was polymerized at 37°C with equimolar taxol.
Plasmid Constructs and Recombinant Proteins

Human moesin and porcine radixin were expressed in *Escherichia coli* as fusion proteins with GST. The cDNA of human moesin in plasmid pGhuMoNcoI (GenSeq accession no. M69866; (Lankes and Furthmayr, 1991)) was digested with NcoI and the fragment containing the moesin coding region was cloned into the pGEZ-KG vector (Guan and Dixon, 1991) to generate plasmid pChMoNcoI. To obtain a missing sequence at the 5’ end of the moesin cDNA, the oligonucleotide 5’-GGGATCCATGCCGAAACCGATCAATG-3’ and 5’-CATTCGATGACGAAACCGATCAATG-3’ was used as the 5’-primer in a PCR reaction to introduce an EcoRI site at the 5’ end of the moesin coding region. The PCR reaction product and pChMoNcoI were double digested with EcoRI and BglII, and the appropriate fragments were reannealed. The resulting construct, plasmid pChMoMut1-1970, contains bp 101 to 1970 of human moesin cDNA, encoding the complete moesin sequence as a GST-fusion protein.

To express porcine radixin as a GST-fusion protein, oligonucleotides 5’-GAGGATCCATGCCGAAACCGATCAATG-3’ and 5’-CATTCGATGACGAAACCGATCAATG-3’ were used to amplify an 1818-bp PCR product of radixin from its cDNA (GenSeq accession no. M64444; Lankes et al., 1993). This PCR product contains the complete coding region of radixin (bp 211-2043) flanked by BamHI and NcoI restriction sites. After digestion with BamHI and NcoI, this fragment was cloned into the corresponding restriction sites of pGEX-KG. E. coli were transformed with the GST-fusion constructs, and the expression of fusion proteins was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). Recombinant GST-fusion proteins were bound to a glutathione-agarose (Sigma Chemical) column and cleaved with thrombin (Sigma Chemical), as described (Guan and Dixon, 1991). Recombinant moesin and radixin were further purified by heparin affinity chromatography, eluted with 300 mM NaCl, 50 mM Tris-HCl, pH 8, and dialyzed into PBS.

Murine Moesin cDNA and Carboxy-terminal Deletion Mutants

A cDNA encoding mouse muscle moesin (GenSeq accession no. M68390) was isolated from a lambda Unizap library (Stratagene, La Jolla, CA) by screening with a 1071-bp PsiI fragment derived from the coding region of human moesin (Lankes and Furthmayr, 1991) and was sub-cloned into pBluescript (Stratagene). The 1945-bp XbaI fragment was excised and cloned into pGEX-KG. The mouse moesin insert in this latter plasmid lacks 33 bp of 5’-coding region. Because human and mouse moesin contain identical predicted amino acid sequences at their amino termini (Lankes et al., 1993), we introduced 33 bp from the 5’ end of the human moesin cDNA in pChMuMut1-1970 into the mouse moesin construct by ligation of appropriate restriction fragments after double digestion with MluI and NcoI. After transection of the resulting constructs into E. coli, GST-moesin fusion proteins are obtained with the correct amino-terminal amino acid sequences, but with various deletions from the carboxy terminus, as described below.

Unidirectional deletions from the 3’ end of the moesin cDNA were generated by digesting linearized plasmid with *EcoRI* for different times. Deletion constructs were treated sequentially with S1 nuclease, the Klenow fragment of DNA polymerase I, and T4 DNA ligase and transfected into competent E. coli. After induction with IPTG, whole bacterial lysates containing GST-moesin fusion proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. Colonies expressing carboxy-terminal deletions of different sizes were picked and further characterized by restriction enzyme analysis and by sequencing from the 3’ end. Bacterial lysates containing selected carboxy-terminal deletion mutants were analyzed for binding to 125I-labeled actin in blot overlays.

**RESULTS**

A meshwork of actin filaments is associated with the cytoplasmic surfaces of glass-adherent neutrophils.
As was reported for rabbit lung polymorphonuclear leukocytes (Boyles and Bainton, 1979; Boyles and Bainton, 1981), we found that the cytoplasmic surfaces of the plasma membranes of circulating bovine neutrophils could be exposed by mechanical cleavage of cells adherent to acid-washed glass cover slips (Figure 1). Adherent fragments of plasma membrane were recognized as cell-sized areas that were not detectable with phase contrast optics (Figure 1, panel a) but were visualized by staining with the fluorescent lipid, 5-[n-tetradecanoylaminofluorescein (Figure 1, panel b). Counter staining with rhodamine-phalloidin showed a coincident pattern of filamentous actin (Figure 1, panel c) reminiscent of the network of globular centers and thin, branched filaments described previously (Boyles and Bainton, 1979; Boyles and Bainton, 1981). This pattern also looks like the actin-based membrane skeletons visualized by similar techniques in erythrocytes, platelets, macrophages, and Dictostelium amoebae (Clarke et al., 1975; Hartwig et al., 1989; Hitt et al., 1994; Stout and Axelrod, 1994), indicating that the neutrophil contains a population of actin filaments in close association with the plasma membrane.

Consistent with this hypothesis, actin co-isolates with a plasma membrane-enriched subcellular fraction (Figure 2, A and B, lane 3), called the γ fraction (Borregaard et al., 1983; Del Buono et al., 1989). In a typical preparation from 1.7 × 10^10 neutrophils, ~39 mg of the γ fraction was recovered. Electron micrographs of this fraction were similar to those obtained with material from human neutrophils (Borregaard et al., 1983; Sengelov et al., 1992) and were consistent with the reported presence of both plasma membrane and mobilizable intracellular vesicles containing plasma membrane markers (Kobayashi and Robinson, 1991; Sengelov et al., 1992). The γ fraction was enriched at least 10-fold in cell surface (Figure 2A, lane 3) and plasma membrane markers (Mottola et al., 1980; Borregaard et al., 1983; Del Buono et al., 1989), and contained a prominent polypeptide identified as actin by M, (Mottola et al., 1980; Stevenson et al., 1989) and by immunoblotting (Stevenson et al., 1989). Actin also was present in whole cell lysates (Figure 2B, lane 1) and cytosol (Figure 2B, lane 2), but little or no silver-stained material was observed at the position of actin in the granule fraction (Figure 2B, lane 4).

The γ fraction is enriched in proteins that bind ^125^I-labeled F-actin in a blot overlay assay (Figure 2C). Major actin-binding polypeptides of 205, 81, and 78 kDa were consistently observed (Figure 2C, lane 3). Other polypeptides of ~67, 69, and 95 kDa were often present (Figure 2C, lane 3), but the numbers and intensities of these proteins varied from preparation to preparation (see below), probably as a result of proteolysis. Relative to the total cell lysate (Figure 2C, lane 1), the 205-kDa polypeptide in the γ fraction was enriched ~15-fold whereas the ~80-kDa doublet was enriched about fivefold (Figure 2C, lane 3). Although the 78- and 81-kDa proteins were also present in the cytosol (Figure 2C, lane 2), none of the major actin-binding proteins cofractionated with the granules (Figure 2C, lane 4). The ratio of actin bound to the 78-kDa protein relative to that at 81-kDa was ~9:1 in cytosol (Figure 2C, lane 2) and ~2:1 in the γ fraction (Figure 2C, lane 3; also see below). Actin binding to the 205-kDa polypeptide varied greatly, but was usually ~10–25% of the total amount bound to γ fraction proteins (Figure 2C, lane 3). Thus, all three actin-binding proteins were associated with plasma mem-

![Figure 1](image-url). Filamentous actin is associated with the cytoplasmic surfaces of bovine neutrophil plasma membranes. Cytoplasmic surfaces were exposed by mechanical unroofing of coverslip-attached cells. Plasma membrane fragments were visualized with (a) phase contrast optics, (b) a fluorescein-lipid dye for membrane bilayers, and (c) rhodamine-phalloidin, a stain for F-actin. The absence of cellular structures detectable in phase (a) indicates that the lipid (b) and actin (c) are associated with cell footprints and not with internal membranes or unroofed, but otherwise intact, cells. Bar, 10 μM.
The actin-binding proteins visualized by the blot overlay technique bind specifically to F-actin (Figure 3). First, binding of $^{125}$I-labeled actin was absolutely dependent on the assembly state of the actin (Figure 3A). At 10 μg/ml, a concentration near the critical concentration for spontaneous polymerization in solution (Wegner, 1982; Bonder et al., 1983), prepolymerized F-actin bound to the 78-, 81-, and 205-kDa proteins and to polypeptides at 67, 69, and 95 kDa (Figure 3A, lane F). Because the ~67-, 69-, and 95-kDa polypeptides were not always observed (for instance, see Figure 3B), their appearance may be dependent on variable amounts of proteolysis. By contrast, 10 μg/ml of monomeric $^{125}$I-labeled G-actin (Figure 3A, lane G) did not bind detectably to any of the polypeptides that bound F-actin. However, trace amounts of a ~87-kDa G-actin-binding polypeptide were observed (Figure 3A, lane G). Denatured, nonpolymerizable actin did not bind any of the actin-binding polypeptides, but exhibited a large nonspecific binding to nitrocellulose (our unpublished observations).

Specificity of binding also was indicated by competition experiments with unlabeled proteins (Figure 3B). In these experiments, unlabeled F-actin competed efficiently with $^{125}$I-labeled F-actin for binding to the 78-, 81-, and 205-kDa polypeptides (Figure 3B, lanes 2 and 3). For instance, a 50-fold excess of unlabeled actin (0.5 mg/ml) reduced the binding of $^{125}$I-labeled F-actin to the ~80-kDa doublet by at least 75%; binding to the 205-kDa protein was reduced by more than an order of magnitude (Figure 3B, lane 2). In the presence of a 250-fold excess of unlabeled F-actin (2.5 mg/ml), the binding of $^{125}$I-labeled F-actin to the ~80-kDa doublet was decreased by more than 95% (Figure 3B, lane 3). By contrast, other electronegative biopolymers at

Figure 2. Proteins biotinylated at the cell surface and F-actin-binding proteins are enriched in a membrane fraction (γ fraction) from bovine neutrophils. Ten micrograms (A and B) or 100 μg (C) of total cell protein (lanes 1), cytosol (lanes 2), plasma membrane-enriched γ fraction (lanes 3), or granule fraction (lanes 4) were electrophoresed on 6–16% gradient polyacrylamide SDS-gels and stained for total protein with silver (B) or blotted onto nitrocellulose and probed with 0.1 μCi/ml $^{125}$I-labeled streptavidin (A) or 50 μg/ml (~50 μCi/mg) $^{125}$I-labeled F-actin (C). The lower edges of the molecular mass standards (lane S), in kDa, are denoted on the left of each panel. Approximate migration positions of a prominent cell surface biotinylated protein (●), actin (*), and major membrane-associated actin-binding proteins of ~205 and ~80 kDa (arrows) are shown to the right of the relevant panels.

Figure 3. Bovine neutrophil membrane proteins bind specifically to $^{125}$I-labeled F-actin in blot overlays. (A) Blot strips of proteins from an isolated γ fraction were incubated with 10 μg/ml $^{125}$I-labeled F-actin (lane F) or 10 μg/ml $^{125}$I-labeled G-actin (lane G). (B) Binding of $^{125}$I-labeled F-actin to blot strips containing a different γ fraction in the absence (lane 1) or presence of 0.5 mg/ml unlabeled F-actin (lane 2), 2.5 mg/ml unlabeled F-actin (lane 3), 2.5 mg/ml taxol-stabilized microtubules (lane 4), 2.5 mg/ml polyaacrylamide (lane 5), 2.5 mg/ml heparin (lane 6), 0.5 M NaCl (lane 7), or 1.0 M NaCl (lane 8). Each lane was loaded with the equivalent of 60 μg membrane protein (~2 × 10⁷ cells). Lines mark the lower edges of the 200-, 97.4-, 69-, and 46-kDa molecular mass standards in the two experiments. Arrows denote the major polypeptides of ~205 and ~80 kDa that bind specifically to F-actin under these conditions. Note also lower molecular mass polypeptides that are often (A), but not always (B), observed.
Figure 4. Myosin S1, but not barbed-end filament-capping proteins, inhibits the binding of 125I-labeled F-actin to the --205-, 81-, and 78-kDa membrane proteins. Blot strips of proteins from an isolated γ fraction were incubated with 10 µg/ml phalloidin-stabilized 125I-labeled F-actin nucleated with 1.250 mol of plasma gelsolin (0.23 µM actin monomers; 0.92 nM filaments). Binding in the absence (lane 1) or presence of 2 mM MgATP (lane 2); 0.14 mg/ml (1.15 µM) myosin S1 (lane 3); myosin S1 plus 2 mM MgATP (lane 4); 1.14 µg/ml (18.1 nM) CapZ (lane 5); or 1.65 µg/ml (18.3 nM) plasma gelsolin (lane 6). Each lane was loaded with the equivalent of 80 µg membrane protein. Lines on the left show the migration positions of the 200- and 97.4-kDa molecular mass standards. Arrows on the right denote the major polypeptides of ~205 and ~80 kDa.

2.5 mg/ml were much less efficient in inhibiting actin binding. Neither microtubules (Figure 3B, lane 4) nor polyaspartic acid (Figure 3B, lane 5) affected F-actin binding to any of the three membrane proteins. Heparin at 2.5 mg/ml reduced the binding of 125I-labeled F-actin to the ~80-kDa doublet by ~25% and the binding to the 205-kDa polypeptide by ~75% (Figure 3B, lane 6). The inhibition by heparin may be due to electrostatic interactions because molar concentrations of NaCl also partially blocked the binding of 125I-labeled F-actin, particularly to the 205-kDa polypeptide (Figure 3B, lanes 7 and 8). However, even 0.5 mg/ml of unlabeled F-actin competed much more efficiently than did 1.0 M NaCl or 2.5 mg/ml of the other acidic biopolymers.

The 205-, 81-, and 78-kDa polypeptides apparently bind to the sides of the actin filaments. First, the 125I-labeled actin used for these experiments was polymerized in the presence of plasma gelsolin under conditions expected to result in stably capped barbed ends (Schwartz and Luna, 1986; Chia et al., 1991). Second, binding of 125I-labeled F-actin to all three of these neutrophil membrane proteins was completely inhibited by a fivefold molar excess (0.14 mg/ml) of myosin S1 (Figure 4, lane 3), a protein with well-characterized binding sites along the sides of actin filaments (Rayment et al., 1993; Schröder et al., 1993). This inhibition was caused by the specific interaction between myosin S1 and actin because MgATP, which dissociates actin-myosin complexes (Fraser et al., 1975), reversed the inhibition by myosin S1 (Figure 4, lane 4). MgATP alone had no detectable effect on the binding of 125I-labeled actin to any of the major actin-binding membrane proteins (Figure 4, lane 2). By contrast, concentrations of CapZ and plasma gelsolin sufficient to block any barbed filament ends that might become exposed during the assay (~20-fold excess over the number of actin filaments or about one capping protein per 12.5 actin monomers) did not inhibit binding of 125I-labeled actin to the 205-, 81-, and 78-kDa membrane proteins (Figure 4, lanes 5 and 6).

Although the identity of the 205-kDa polypeptide is presently unknown, the major ~80-kDa actin-binding doublet consists of moesin and ezrin (Figure 5A). In both HeLa cells (Figure 5A, lane 1) and bovine neutrophil membranes (Figure 5A, lane 2), the 78-kDa and 81-kDa polypeptides comigrated with proteins that were immunoreactive with antibodies against moesin.

Figure 5. Moesin and ezrin constitute the major ~80-kDa protein doublet that binds F-actin on blot overlays. Although radixin is not present in neutrophils in significant quantities, it also binds actin. (A) Binding of 125I-labeled F-actin (F-actin); monoclonal anti-moesin mAb38/87 (α-M); polyclonal anti-ezrin pAsE (α-E); and polyclonal anti-radixin pAsR (α-R) to proteins from a whole cell lysate of 4 × 10^5 HeLa S3 cells (lane 1), 40 µg of an isolated bovine neutrophil membrane γ fraction (lane 2), a Triton X-100 extract from 40 µg of the γ fraction (lane 3), or to proteins immunoprecipitated from this Triton extract with pAsR (lane 4), pAsE (lane 5), or affinity-purified anti-moesin pAbMo (lane 6). Only the ~65- to ~90-kDa regions of the nitrocellulose blots are shown. Note the binding of F-actin, mAb38/87, and pAsR to radixin in HeLa cells and the apparent absence of significant amounts of radixin in neutrophil membranes. (B) F-actin blot overlay of recombinant radixin (lane 1) and recombinant moesin (lane 2).
(α-M) and ezrin (α-E), respectively. Although present in HeLa cells (Figure 5A, lane 1), the related protein radixin was not detectable by immunoblotting (α-R) of bovine neutrophils (our unpublished observations) or membranes (Figure 5A, lane 2). After solubilization (Figure 5A, lane 3) and immunoprecipitation with antibodies specific for radixin (Figure 5A, lane 4), ezrin (Figure 5A, lane 5), or moesin (Figure 5A, lane 6), the immunoprecipitated ezrin and moesin bound to \(^{125}\text{I}\)-labeled F-actin on blot overlays (Figure 5A, F-actin, lanes 5 and 6). Although radixin was not present in neutrophils in significant quantities, bacterially expressed radixin also bound directly to actin on blot overlays (Figure 5B, lane 1), an activity shared with recombinant moesin (Figure 5B, lane 2). Thus, moesin is the 78-kDa protein responsible for most of the observed binding at \(~80\) kDa, and ezrin is the 81-kDa polypeptide that comprises the lesser F-actin binding component of the \(~80\)-kDa doublet.

Consistent with previous observations in other cell types (Amieva et al., 1994), immunofluorescence microscopy with moesin-specific polyclonal antibodies (Amieva, unpublished observation) and with anti-moesin mAb38/87 (Figure 6) suggested that moesin is concentrated at the cytoplasmic surface of the neutrophil plasma membrane. Although mAb38/87 also recognized radixin on immunoblots (Figure 5A, α-M, lane 1), the absence of radixin from bovine neutrophils (Figure 5A, lanes 2 and 4) ensured the specificity of mAb38/87 in these cells. Bright ring fluorescence was observed in neutrophils permeabilized with Triton after fixation (Figure 6a), but not in most unpermeabilized cells (Figure 6c). The few unpermeabilized cells that did stain for moesin (Figure 6c, arrow) usually appeared to have been damaged during processing (Figure 6d, arrow). Thus, as in most other cell types (Lankes and Furthmayr, 1991; Berryman et al., 1993; Franck et al., 1993; Amieva et al., 1994), moesin in peripheral blood neutrophils is a membrane-associated cytoplasmic protein, rather than an extracellular protein bound to heparin or to proteoglycans (Lankes et al., 1988).

As suggested by its direct binding to filamentous actin, moesin co-localized with F-actin on the cytoplasmic surfaces of plasma membrane fragments exposed by mechanical unroofing of cells (Figure 7). Moesin staining on these isolated plasma membrane fragments exhibited an intensely fluorescent punctate pattern superimposed on regions with a fainter, more diffuse fluorescence (Figure 7a). Actin filaments exhib-
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Figure 7. Moesin co-associates with actin on the cytoplasmic surfaces of membrane fragments from mechanically unroofed bovine neutrophils. Plasma membrane fragments were visualized by immunofluorescence microscopy using (a) monoclonal anti-moesin mAb38/87 and (b) fluorescein-phalloidin. Note the absence of intracellular structures detectable by phase optics (c). Bar, 10 μm.

ited an essentially identical pattern (Figure 7b), indicating co-localization with moesin at this level of resolution. Because the mechanically unroofed plasma membrane fragments were uncontaminated by granules or other intracellular membranes (Figure 7c), these results demonstrate that moesin, as well as actin, associates directly or indirectly with neutrophil plasma membranes. These results also are consistent with moesin localization in microvilli, membrane ruffles, and other cell surface projections (Berryman et al., 1993; Franck et al., 1993; Armieva et al., 1994).

The carboxy-terminal 22 residues of moesin are required for its binding to [125I]-labeled F-actin on blot overlays (Figure 8). Analysis of bacterially expressed moesin and a series of carboxy-terminal deletion constructs (Figure 8A) showed that only the full-length molecule (Figure 8B, lane 1) bound to [125I]-labeled F-actin on blot overlays (Figure 8C, lane 1). Although not studied here, the homologous region of ezrin also has recently been shown to bind F-actin in a column chromatography assay (Turunen et al., 1994). Thus, the carboxy termini of the ezrin-radixin-moesin gene family, which are highly conserved among all family members (Figure 9), apparently constitute specific sites for direct, high-affinity binding to filamentous actin.

**DISCUSSION**

The results shown here suggest that neutrophil plasma membranes contain an actin-based membrane skeleton, which includes moesin and ezrin. In agreement with previous reports of a subplasmalemmal filament complex (Boyles and Bainton, 1979; Boyles and Bainton, 1981), we find that actin-containing meshworks are tightly associated with the cytoplasmic surfaces of neutrophil plasma membranes (Figure 1). Furthermore, actin and actin-binding proteins identifiable by F-actin blot overlays co-purify with plasma membranes on Percoll density gradients (Figure 2). Prominent actin-binding membrane-associated proteins of 78, 81, and 205 kDa bind specifically to filamentous actin, and not to monomeric actin or to other acidic biopolymers (Figure 3). Binding of F-actin to these proteins is blocked by myosin S1, but is unaffected by proteins that cap barbed filament ends (Figure 4), suggesting that, like myosin, the membrane proteins associate laterally with the actin filaments.

The ~80-kDa protein doublet consists of moesin and ezrin (Figure 5A), proteins that, until recently (Turunen et al., 1994), were not known to bind directly to actin. Neutrophil moesin is highly enriched at the plasma membrane (Figures 2 and 6) and co-localizes with the filamentous actin that partitions with the plasma membrane after mechanical unroofing (Figure 7). Although radixin is not present in significant quantities in bovine neutrophils, this third member of the highly conserved ezrin-radixin-moesin gene family also binds directly to F-actin (Figure 5B). This observation is consistent with the localization of the actin-binding site to a highly conserved sequence at the extreme carboxy terminus of moesin (Figures 8 and 9). Thus, our results with actin blot overlays are in agreement with the recent report that GST fusion proteins containing the carboxy-terminal 34 amino acids of ezrin bind F-actin (Turunen et al., 1994).

Although preparations of radixin have been reported to cap the barbed ends of actin filaments (Tsukita et al., 1989), the F-actin binding observed in our experiments is mediated by binding to the sides of the filaments. First, binding was observed to actin filaments with stably capped barbed ends (Figures 2–5). Further, at the critical concentration for actin assem-
Figure 8. Truncation of the moesin carboxy terminus by 22 amino acids results in complete loss of 125I-labeled F-actin binding on blot overlays. (A) Schematic depiction of the moesin deletions that were expressed as GST-fusion proteins 1 through 6 and analyzed below. The sites of the truncation and the newly created carboxy-terminal sequences also are indicated. Numbers refer to amino acid positions (AA) in the moesin amino acid sequence. (B) Coomassie blue-stained SDS-gel of lysates from bacteria expressing GST-fusion proteins 1 through 6. Numbers on the left denote their respective molecular masses in kilodaltons. (C) 125I-labeled F-actin blot overlay of the lysates shown in B. Only full-length moesin binds to actin (lane 1).

Figure 9. Sequence similarities of the carboxy-terminal actin-binding sites in moesin, radixin, and ezrin. Identical residues are indicated by dots. The proteins, with databank accession numbers in parentheses, are as follows: human moesin (M69066), porcine radixin (M86444), bovine ezrin (M98498), and human ezrin (P15311). Required amino acids in moesin (this study) and ezrin (Turunen et al., 1994) are marked by a heavy overline. A sequence in human ezrin that has been shown to be sufficient for actin-binding activity (Turunen et al., 1994) is underlined.

Our results also support the previous suggestion that the actin-binding site is masked in soluble, full-length ezrin by the presence of the amino-terminal domain (Algrain et al., 1993). In contrast to the actin overlay experiments presented here, neither purified ezrin (Bretscher, 1983; Krieg and Hunter, 1992; Fazioli et al., 1993) nor moesin extracted from neutrophils (Pestonjamsap, Strassel, and Luna, unpublished observations) binds directly to F-actin in solution at physiological salt concentrations. Because moesin and ezrin from cytosol do bind F-actin on blot overlays (Figure 2, lane 2), it appears that the actin-binding sites are present in these proteins but are blocked unless exposed by truncation (Algrain et al., 1993) or SDS-denaturation (this study) of amino-terminal sequences. This hypothesis is supported by the study of Turunen et al. (1994), in which the actin-binding GST-ezrin fusion proteins all lacked the ezrin amino-terminus (amino acids 1–242).

The reported co-localizations of actin with moesin, ezrin, and radixin at membrane surfaces suggests that the actin-binding site may be unmasked in vivo by a conformational change associated with binding to the membrane. Such a conformational change could be a direct consequence of attachment to membrane proteins or could result from dimerization (Gary and Bretscher, 1993; Andréoli et al., 1994) or phosphorylation (Gould et al., 1986; Bretscher, 1989; Hanzel et al., 1991; Fazioli et al., 1993) at the membrane surface.

Although the physiological role of the carboxy-terminal actin-binding site in moesin, ezrin, and radixin is difficult to evaluate at present, it is clear that only a
subset of proteins capable of binding F-actin in vivo bind to F-actin in our blot overlay assay. For instance, neither myosin, spectrin, nor a number of other cytoplasmic actin-binding proteins bind under our assay conditions (our unpublished observations). So far, the only other identified proteins that are positive in this assay are the transmembrane actin-binding protein, ponticulin, and the filopodial actin-bundling protein known as p30a (Chia et al., 1991; Fechheimer and Furukawa, 1993). Because both ponticulin (Hitt et al., 1994) and p30a (Fechheimer et al., 1994) mediate actin filament assembly and/or stability at the membrane surface, it is tempting to speculate that the blot overlay assay selectively identifies actin-binding proteins that can associate with membranes. Perhaps proteins that function in or at hydrophobic bilayer interiors contain actin-binding sites that are more resistant to the denaturing effects of SDS and other detergents. In any case, the absence of any significant sequence similarities between the moesin carboxy terminus and either ponticulin or p30a indicates that more than one type of actin binding site is involved. However, it may be noteworthy that amino acids 360–374 in ezrin are 87% identical to residues 196–210 in p30a (Fechheimer et al., 1991), an actin-binding protein concentrated in cell surface extensions, cleavage furrows, and sites of cell-cell interactions in Dictyostelium discoideum (Fechheimer, 1987; Fechheimer et al., 1994; Furukawa and Fechheimer, 1994).

Given the proteins so far identified by the F-actin blot overlay assay, we suggest that the ~205-kDa actin-binding protein (Figures 2 and 3) may be another component of the neutrophil membrane skeleton. This hypothesis is consistent with its striking enrichment with plasma membranes (Figure 2) and with detergent-insoluble cytoskeletons (our unpublished observations). Based on preliminary immunoassays, this protein is not myosin, fodrin, talin, or tensin and thus may be a novel membrane-associated actin-binding protein. Future work will utilize the large amounts of membranes obtainable from bovine neutrophils to isolate new membrane skeleton components and to characterize their interactions with each other and with the plasma membrane. The ultimate goal is to determine the roles of membrane skeletal proteins in the biological functions of neutrophils and other motile cells.

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Note added in proof. Following the acceptance of this paper, we tested additional GST-moesin fusion proteins for their ability to bind 125I-labeled F-actin on blot overlays. In agreement with the results summarized in Figure 9, we found that the carboxy-terminal 48 amino acids of moesin are sufficient for F-actin binding.

REFERENCES


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