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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 ^{125}I-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 ^{125}I-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 ^{125}I-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 chemotactic 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 β_2-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 (Amieva *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 ^{125}I-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 myosin 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 LKB Biotechnology, Piscataway, NJ). 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 KH_2PO_4, 0.63 mM Na_2HPO_4, 5.55 mM glucose, 4.17 mM $NaHCO_3$, 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 $\times$ 10^8 cells/ml in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 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 $\times$ *g* for 10 min, and the supernatant was spun through a cushion of Percoll, which had been adjusted to a density of 1.050 with relaxation buffer, at 46,000 $\times$ *g* for 35 min. The cytosol, plasma membrane fraction, and granule fraction were harvested and recentrifuged at 140,000 $\times$ *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 KH_2PO_4, 0.63 mM Na_2HPO_4, 5.55 mM glucose, 4.17 mM $NaHCO_3$, 10 mM HEPES, pH 7.4; PHEM buffer, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9; TBST, 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 10^8 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 γ fraction was prepared as above. Protein concentrations were determined in the presence of 1% sodium dodecyl sulfate (SDS) by the method of Lowry *et al.* (1951), 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 ^{125}I-labeled streptavidin (Goodloe-Holland and 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 pAs**E** (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 pAs**R** (Amieva *et al.*, 1994), prepared against recombinant human radixin, is specific for radixin both in immunoblotting and immunoprecipitations (see below). Rabbit polyclonal anti-moesin pAs**Mo**ER (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 pAb**Mo** is specific for moesin both by immunoblotting and by immunoprecipitation (Amieva and Furthmayr, unpublished observations). Monoclonal anti-moesin mAb**38/87**, 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 × 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 $CaCl_2$, 1.0 mM $MgCl_2$, 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 $MgCl_2$, pH 6.9 (PHEM buffer; Schliwa 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 [PHEM 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 6 U/ml 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 U/ml of fluorescein-phalloidin (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 mAb**38/87** (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/Organon Teknika, Durham, NC). Coverslips were washed and mounted as described above.

Immunofluorescence. Neutrophils were isolated and allowed to attach to acid-cleaned 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-morpholino]ethanesulfonate (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 mAb**38/87** 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 (M_r) were determined by comparison with prestained standards (Amersham Corporation, Arlington Heights, IL). The bottom edges of these standards are assumed to approximate the M_r of the unlabeled proteins.

Column-purified rabbit muscle G-actin was labeled with ^{125}I-Bolton Hunter reagent (Dupont NEN, Wilmington, DE) and stored in dialysis (Schwartz and Luna, 1986; Wuestehube and Luna, 1987). For blot overlays with F-actin, ^{125}I-labeled actin was polymerized in the presence of rabbit plasma gelsolin, stabilized with phalloidin, and stored at 1 mg/ml on ice (Chia *et al.*, 1991). Unless stated otherwise, phalloidin-stabilized ^{125}I-labeled F-actin was diluted to a final concentration of 50 μg/ml with 5% (wt/vol) nonfat powdered milk (Carnation Co., Los Angeles, CA), 5 μM phalloidin (Boehringer Mannheim, Indianapolis, IN) in TBST before incubation with nitrocellulose blots for 2 h at ~21°C (Chia *et al.*, 1991). Blot overlays with ^{125}I-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 phalloidin and ATP were limited to their roles in stabilizing actin polymer and monomer, respectively (our unpublished observations).

Competition with Unlabeled Proteins

Proteins. Polyaspartic acid (15–50 kDa) and heparin (sodium salt, Grade II) were purchased from Sigma Chemical. Phosphocellulose-purified tubulin (Williams and Lee, 1982), a generous gift from Dr. Curtis Wilkerson (Worcester Foundation for Experimental Biology, Shrewsbury, MA), was polymerized at 37°C with equimolar taxol

immediately before use. Myosin subfragment 1 (S1) was generated by chymotryptic digestion of rabbit muscle myosin and was purified on DEAE-cellulose, as described previously (Weeds and Pope, 1977; Luna *et al.*, 1982). Myosin S1 was stored for ≤2 wk at 0°C in 80 mM KCl, 50 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1% ethanol, pH 7.0. CapZ was generously supplied by Dr. John A. Cooper (Washington University School of Medicine, St. Louis, MO). Plasma gelsolin was prepared from frozen rabbit plasma (Pel-Freez Biologicals, Rogers, AR) by ammonium sulfate fractionation and chromatography on DEAE-Sepharose, Sephacryl S-200, and Blue-Sepharose (Chaponnier *et al.*, 1985; Soua *et al.*, 1985; Cooper *et al.*, 1987).

Procedures. For experiments with unlabeled actin and other acidic biopolymers, competition solutions were prepared in 50 mM KCl, 2 mM $MgCl_2$, 80 μM phalloidin, 0.16 mM CaATP, 0.16 mM dithiothreitol, 0.02% sodium azide, 20 mM PIPES, pH 7.0. Unlabeled actin filaments were nucleated with a 1:800 mole ratio of plasma gelsolin, and solutions with microtubules contained 20 μM taxol. Solutions containing either unlabeled F-actin, taxol-stabilized microtubules, polyaspartic acid, or heparin were diluted with an equal vol of 10% milk, TBST and incubated for 1 h at 21°C with pre-blocked strips of nitrocellulose, each containing ~60 μg of membranes. ^{125}I-labeled F-actin was then added to a final concentration of 10 μg/ml, and the incubation was continued for another hour.

For experiments with myosin S1 and other actin-binding proteins, competition solutions were prepared in 56 mM KCl, 1.6 mM $MgCl_2$, 0.8 μM phalloidin, 130 μM $CaCl_2$, 10 mM imidazole, 16 mM PIPES, pH 7.0. Where indicated, these solutions also contained 4 mM MgATP. Competition solutions containing myosin S1, CapZ, or plasma gelsolin were mixed with 10 μg/ml ^{125}I-labeled F-actin that had been nucleated with a 1:250 mole ratio of plasma gelsolin, i.e., filaments contained an average of 250 monomers. After a 30-min pre-incubation, the solutions were diluted with an equal vol of 10% milk, TBST and added to blot strips, each containing ~80 μg of membranes. The incubation was continued for another hour at 21°C, and then the blot strips were rinsed five times for 5 min, air dried, and exposed to XAR-2 film (Eastman Kodak Co., Rochester, NY) at −85°C in the presence of a Cronex Lightening Plus intensifying screen (Dupont). Relative amounts of bound radioactivity were quantified with a PhosphorImager (Molecular Dynamics).

Immunoprecipitation

Membranes (1 mg) were extracted on ice with 1 ml of 1% Triton X-100, 250 mM NaCl, 5 mM EDTA, 25 mM Tris, pH 7.5 (Firestone and Winguth, 1990), which also contained the following protease inhibitors: 5 mM 4-(2-aminoethyl)benzene sulfonyl fluoride (Calbiochem, La Jolla, CA), 1 mM benzamidine (Sigma Chemical), 10 μg/ml chymostatin, 10 μM leupeptin, 5 μM pepstatin, 2 mM *N*-CBZ-phenylalanine, and 1 mM phenylmethylsulfonyl fluoride. After clarification at 200,000 × *g* for 30 min at 4°C, membrane extracts were absorbed with nonspecific IgG on protein A-agarose (Bio-Rad). Portions (160 μl) were then incubated with 16 μl pAs**R** serum, 16 μl pAs**E** serum, or 21 μg of pAb**Mo** for 15 min at 4°C, and then with protein A-agarose overnight at 4°C. The agarose beads were centrifuged (13,000 × *g*, 5 min, 21°C) through 1 M sucrose, resuspended in Laemmli sample buffer (Laemmli, 1970), and denatured as described above.

Denatured immunoprecipitates were electrophoresed into 7.5% polyacrylamide SDS gels, blotted onto nitrocellulose, and incubated either with ^{125}I-labeled F-actin or with pAs**R** serum, pAs**E** serum, or mAb**38/87** IgG in 3% BSA, TBST. The mAb**38/87** blots then were incubated with 5 μg/ml rabbit anti-mouse IgG, specific for the Fc domain (Pierce), before a 1-h incubation with 0.1 μCi/ml ^{125}I-labeled protein A (Dupont). Blots were washed, dried, and exposed to film, as described above.

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 UIII (GenSeq accession no. M69066; (Lankes and Furthmayr, 1991)) was digested with *Nco*I and the fragment containing the moesin coding region was cloned into the pGEZ-KG vector (Guan and Dixon, 1991) to generate plasmid pGhuMo*Nco*I. To obtain a missing sequence at the 5′ end of the moesin cDNA, the oligonucleotide 5′-GGAATTCATATGC-CCAAAACGATCAGTGTG-3′ was used as the 5′-primer in a PCR reaction to introduce an *Eco*RI site at the 5′ end of the moesin coding region. The PCR reaction product and pGhuMo*Nco*I were double digested with *Eco*RI and Bgl2, and the appropriate fragments were reannealed. The resulting construct, plasmid $pGhuMo_{101-1970}$, 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′-CATTC-CATGGCATCTGCAAAGGC-3′ were used to amplify an 1818-bp PCR product of radixin from its cDNA (GenSeq accession no. M86444; Lankes *et al.*, 1993). This PCR product contains the complete coding region of radixin (bp 211-2043) flanked by *Bam*HI and *Nco*I restriction sites. After digestion with *Bam*HI and *Nco*I, this fragment was cloned into the corresponding restriction sites of pGEX-KG.

E. coli were transfected 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. M86390) was isolated from a lambda Unizap library (Stratagene, La Jolla, CA) by screening with a 1071-bp *Pst*I fragment derived from the coding region of human moesin (Lankes and Furthmayr, 1991) and was sub-cloned into pBluescript (Stratagene). The 1945-bp *Xba*I 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 $pGhuMo_{101-1970}$ into the mouse moesin construct by ligation of appropriate restriction fragments after double digestion with Mlu1 and *Nco*I. After transfection 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 *Exo*III 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 ^{125}I-labeled actin in blot overlays.

RESULTS

A meshwork of actin filaments is associated with the cytoplasmic surfaces of glass-adherent neutrophils

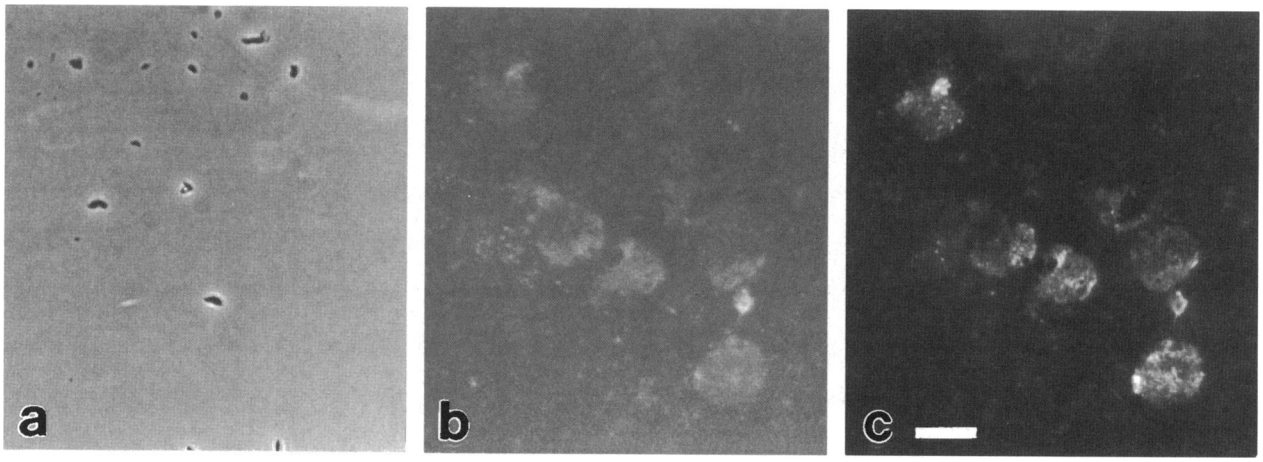

Figure 1. Filamentous actin is associated with the cytoplasmic surfaces of bovine neutrophil plasma membranes. Cytoplasmic surfaces were exposed by mechanical unroofing of coverslip-attached cells. Plasma membrane fragments were visualized with (a) phase contrast optics, (b) a fluorescein-lipid dye for 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.

(Figure 1). 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 in circulating bovine neutrophils could be exposed by mechanical cleavage of cells adherent to acid-washed glass coverslips (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*-tetradecanoyl]aminofluorescein (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 *Dictyostelium* 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; Sengeløv *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; Sengeløv *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_r (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-

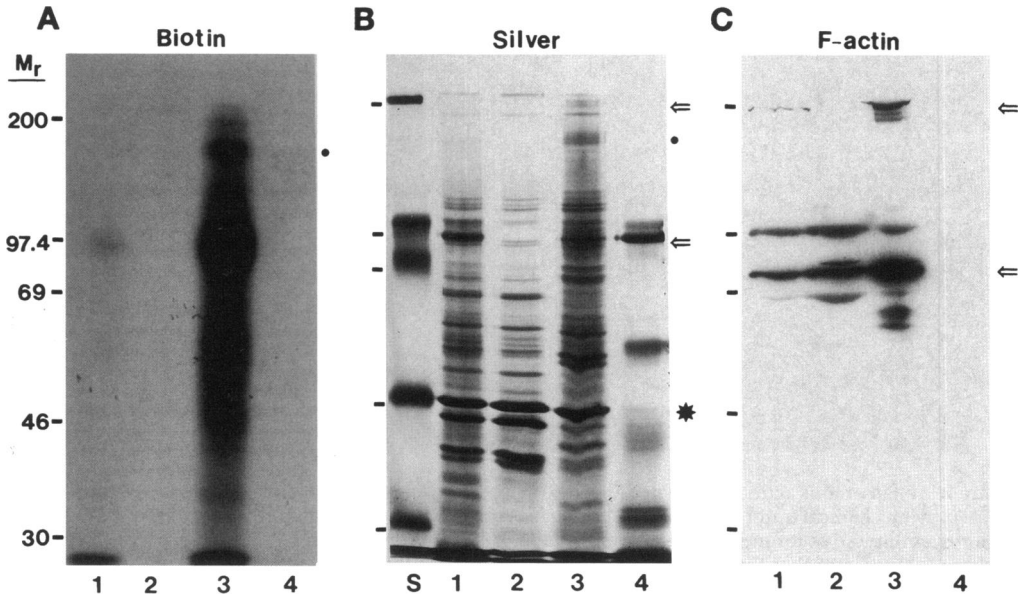

Figure 2. Proteins biotinylated at the cell surface and F-actin–binding proteins are enriched in a membrane fraction (γ fraction) from bovine neutrophils. Ten micrograms (A and B) or 100 μg (C) of total cell protein (lanes 1), cytosol (lanes 2), plasma membrane-enriched γ fraction (lanes 3), or granule 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.

branes, with the greatest enrichment observed for the 205-kDa protein.

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

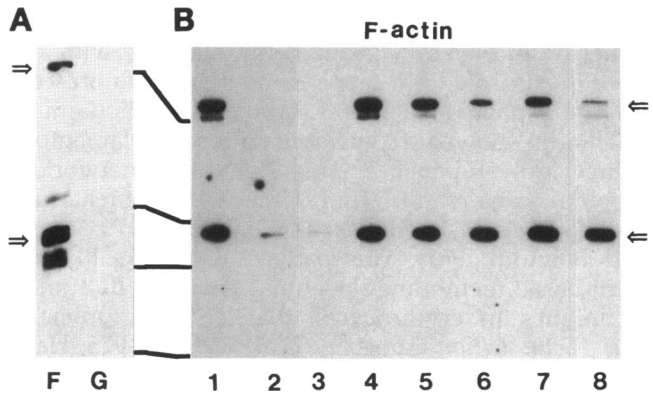

Figure 3. Bovine neutrophil membrane proteins bind specifically to ^{125}I-labeled F-actin in blot overlays. (A) Blot strips of proteins from an isolated γ fraction were incubated with 10 μg/ml ^{125}I-labeled F-actin (lane F) or 10 μg/ml ^{125}I-labeled G-actin (lane G). (B) Binding of ^{125}I-labeled 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 polyaspartic acid (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 × 10^7 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.

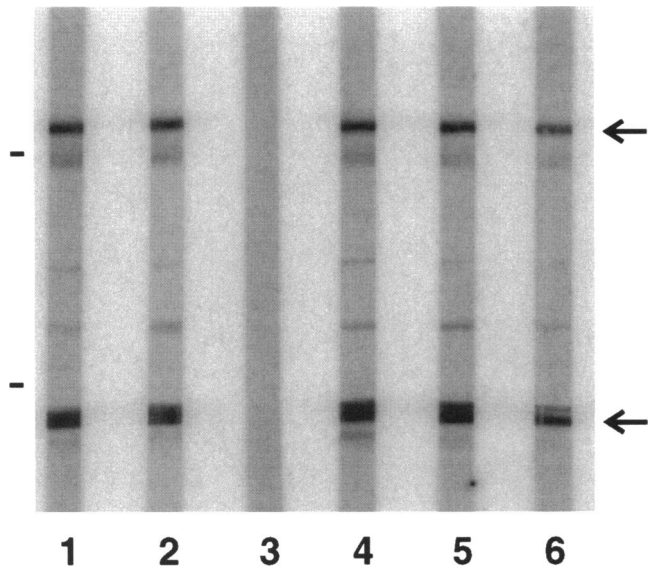

Figure 4. Myosin S1, but not barbed-end filament-capping proteins, inhibits the binding of ^{125}I-labeled F-actin to the ~205-, 81-, and 78-kDa membrane proteins. Blot strips of proteins from an isolated γ fraction were incubated with 10 μg/ml phalloidin-stabilized ^{125}I-labeled F-actin nucleated 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 at 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 ^{125}I-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 ^{125}I-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 ^{125}I-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 ^{125}I-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 ^{125}I-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 ^{125}I-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

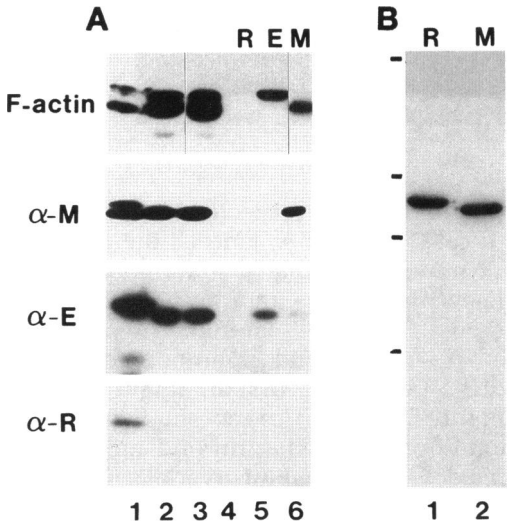

Figure 5. Moesin and ezrin constitute the major ~80-kDa protein doublet that binds F-actin on blot overlays. Although radixin is not present in neutrophils in significant quantities, it also binds actin. (A) Binding of ^{125}I-labeled F-actin (F-actin); monoclonal anti-moesin mAb**38/87** (α-M); polyclonal anti-ezrin pAs**E** (α-E); and polyclonal anti-radixin pAs**R** (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 pAs**R** (lane 4), pAs**E** (lane 5), or affinity-purified anti-moesin pAb**Mo** (lane 6). Only the ~65- to ~90-kDa regions of the nitrocellulose blots are shown. Note the binding of F-actin, mAb**38/87**, and pAs**R** 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).

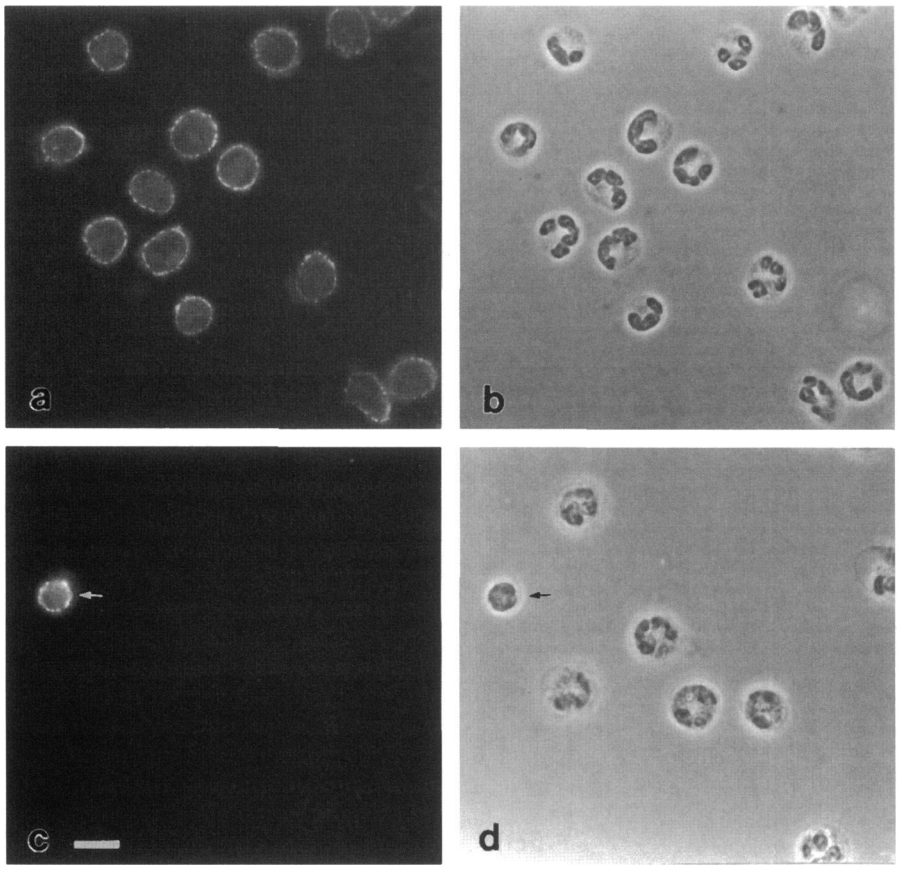

Figure 6. Immunofluorescence microscopy with anti-moesin antibody mAb**38/87** shows that moesin is concentrated at or near the inner surface of the neutrophil plasma membrane. Glass-attached neutrophils were stained with monoclonal anti-moesin with (a and b) and without (c and d) detergent permeabilization. Fluorescence (a and c) and phase (b and d). Bar, 10 μm. Most nonpermeabilized cells that stain with anti-moesin are obviously damaged (c and d; arrows). No fluorescence signal was observed in the absence of the primary antibody (our unpublished observations).

(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}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 mAb**38/87** (Figure 6) suggested that moesin is concentrated at the cytoplasmic surface of the neutrophil plasma membrane. Although mAb**38/87** 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 mAb**38/87** 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 proteoheparan sulfates (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-

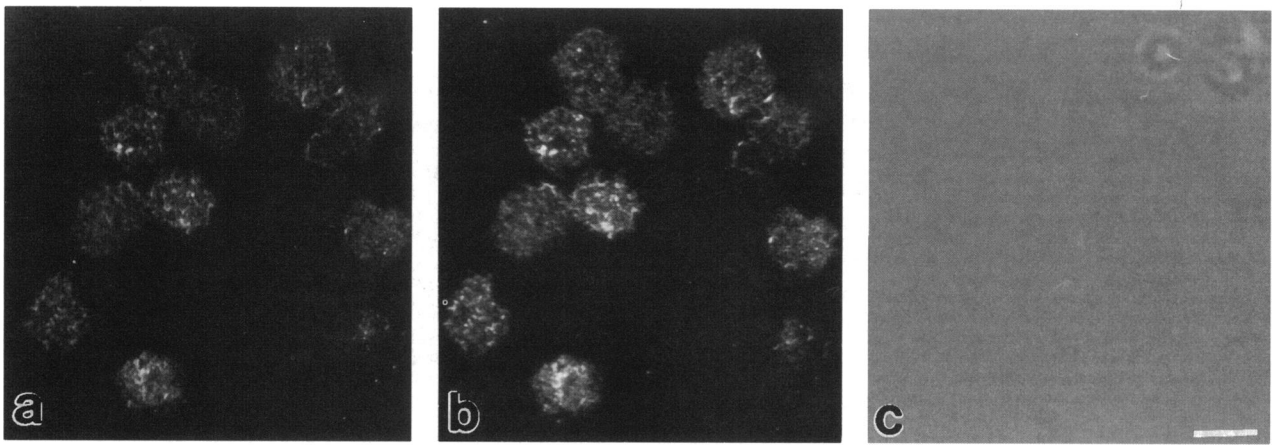

Figure 7. Moesin co-associates with actin on the cytoplasmic surfaces of membrane fragments from mechanically unroofed bovine neutrophils. Plasma membrane fragments were visualized by immunofluorescence microscopy using (a) monoclonal anti-moesin mAb**38/87** and (b) fluorescein-phalloidin. Note the 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; Amieva *et al.*, 1994).

The carboxy-terminal 22 residues of moesin are required for its binding to ^{125}I-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 ^{125}I-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-

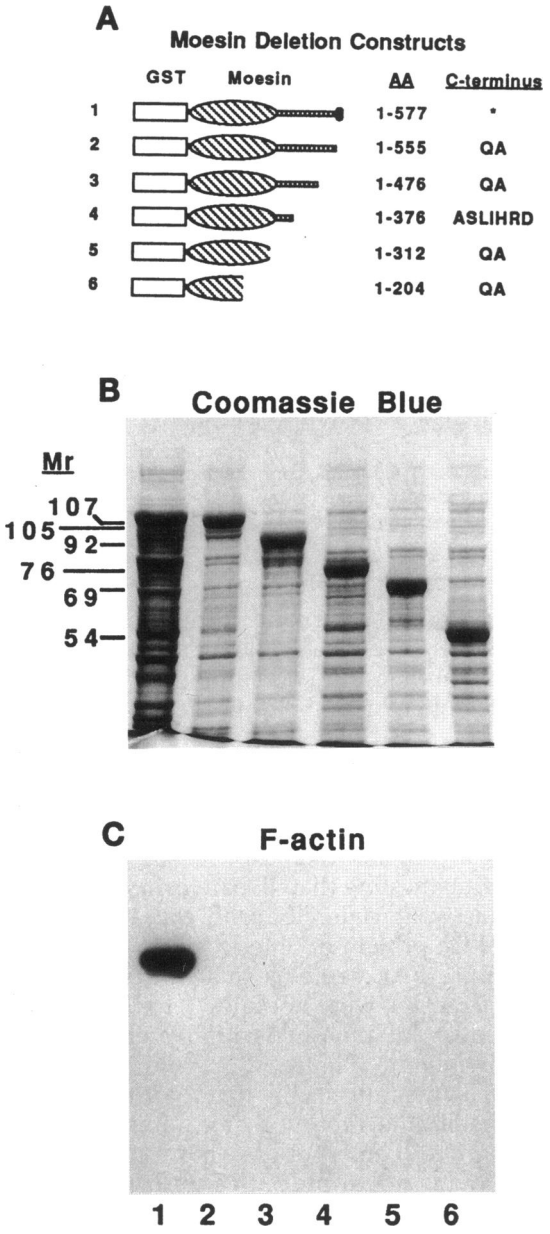

Figure 8. Truncation of the moesin carboxy terminus by 22 amino acids results in complete loss of ^{125}I-labeled F-actin binding on blot overlays. (A) Schematic depiction of the moesin deletions that were expressed as GST-fusion proteins 1 through 6 and analyzed below. The sites of the truncation and 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) ^{125}I-labeled F-actin blot overlay of the lysates shown in B. Only full-length moesin binds to actin (lane 1).

bly, no binding of polymerization-competent actin monomers to ezrin or moesin was observed although a weak interaction was seen with a protein similar in

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h-moesin   IHAENMRLGRDKYKTLRQIRQGNTKQRIDEFESM  577
p-radixin  L····VKA·····················A·  583
b-ezrin    ··N····Q·····················A·  580
h-ezrin    ··N····Q·····················AL  585
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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.

size to gelsolin (Figure 3A, lane G). Finally, binding was not significantly affected by excess levels of the barbed end capping proteins, CapZ and plasma gelsolin, but was inhibited by myosin S1, which binds along the sides of actin filaments (Figure 4). Thus, our results support the many studies that show ezrin, radixin, or moesin to be localized along the lengths of cell surface structures in an apparently lateral association with actin filaments (Bretscher, 1983; Gould *et al.*, 1986; Pakkanen *et al.*, 1987; Goslin *et al.*, 1989; Sato *et al.*, 1992; Berryman *et al.*, 1993).

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 (Pestonjasmasp, 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 ^{125}I-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.

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