Purification and Characterization of an $\alpha_1\beta_2$ Isoform of CapZ from Human Erythrocytes: Cytosolic Location and Inability To Bind to Mg^{2+} Ghosts Suggest That Erythrocyte Actin Filaments Are Capped by Adducin[†]

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ABSTRACT: CapZ ("capping protein") is a heterodimeric actin capping protein that blocks actin filament assembly and disassembly at the fast growing (barbed) filament ends and is proposed to function in regulating actin filament dynamics as well as in stabilizing actin filament lengths in muscle and nonmuscle cells. We show here that erythrocytes contain a nonmuscle isoform of capZ (EcapZ) that is present exclusively in the cytosol and is not associated with the short actin filaments in the erythrocyte membrane skeleton. This is unlike other cell types where capZ is associated with cytoskeletal actin filaments and suggests that cytosolic EcapZ may be inactive, or alternatively, that the barbed ends are capped by adducin, a membrane skeleton protein that was shown recently to cap actin filament barbed ends *in vitro* [Kuhlman, P. A., Hughes, C. A., Bennett, V., & Fowler, V. M. (1996) *J. Biol. Chem. 271*, 7986]. To distinguish between these possibilities, we purified EcapZ from erythrocyte cytosol and characterized its biochemical and functional properties. Two-dimensional gel electrophoresis and western blotting reveals the EcapZ subunit composition to be $\alpha_1\beta_2$, as described for capZ from many other nonmuscle cells, with no evidence for posttranslational modifications. Purified EcapZ is fully functional in blocking actin elongation from barbed filament ends (K_{cap} ∼1−5 nM) as well as in nucleating actin polymerization. Furthermore, cytosolic EcapZ binds to actin filament barbed ends, indicating that sequestering of EcapZ by a cytosolic inhibitory factor or insufficient amounts of EcapZ in cytosol also cannot account for its absence from the membrane skeleton. To test directly whether the barbed ends of the erythrocyte actin filaments were already capped, we measured binding of purified EcapZ to isolated membranes. Purified EcapZ does not cosediment with membranes prepared by hypotonic lysis in the presence of magnesium, suggesting that the barbed ends of the erythrocyte actin filaments are capped under these conditions but not by EcapZ. In contrast, purified EcapZ stoichiometrically reassociates with all the actin filament barbed ends in membranes prepared by hypotonic lysis in 5 mM sodium phosphate, pH 8.0 (5P8), conditions in which the barbed filament ends were previously reported to be uncapped. Comparison of the amounts of adducin associated with membranes prepared in the presence and absence of magnesium reveals that 60−80% of the adducin dissociates from the membrane during hemolysis and washing in 5P8 buffer, suggesting that the barbed ends become artifactually uncapped due to loss of adducin. The erythrocyte actin filaments may thus represent a specialized class of membrane-associated actin filaments that are capped by adducin instead of capZ.

The ability to control the exchange of actin monomers from the fast-growing (barbed) and slow-growing (pointed) ends of actin filaments is one means by which cells regulate actin filament lengths and control the formation of specialized actin filament networks that are tailored for specific physiological functions (*1*, *2*). Actin filament networks range from the semicrystalline packing of long actin filaments into parallel bundles in microvilli to the antiparallel arrays of long actin filaments observed in nonmuscle stress fibers and in striated muscle sarcomeres to the more loosely packed, isotropic networks of shorter filaments in the cortical region of the cell. A particularly well-studied example of the latter is the human erythrocyte membrane skeleton, where short actin filaments (33 ± 5 nm) (*3*) are cross-linked by long, flexible spectrin molecules into a quasihexagonal network attached to the cytoplasmic surface of the plasma membrane. The organization and connectivity of this spectrin−actin network is thought to be an important determinant of the mechanical properties of the erythrocyte *in vivo* (*4*). In these specialized cells, unlike other nonmuscle cells, all of the short actin filaments in the spectrin membrane skeleton are closely associated with the plasma membrane, and none extend into the cytoplasm.

The ability to isolate large quantities of highly purified plasma membrane preparations from erythrocytes, together with the abundance of actin filament ends relative to filament length in the membrane skeleton, makes the erythrocyte an excellent model system for biochemical characterization of components regulating actin filament length. In addition, the narrow gaussian distribution of actin filament lengths in the membrane skeleton indicates that both filament ends are

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likely to be capped in order to prevent monomer association and dissociation and redistribution of filament lengths (for a more extensive discussion see refs *5*, and *6*). The actin filament pointed end capping protein in erythrocytes is well-established to be tropomodulin (*7*). Tropomodulin is also associated with the pointed (free) ends of thin filaments in striated muscle, where it functions *in vivo* to maintain thin filament length (*8*; for a review, see ref *5*). The identity of the actin filament barbed end capping protein in erythrocytes is less certain. Recently, we have identified a novel barbed end capping activity associated with purified adducin (*9*), an erythrocyte membrane skeleton protein that promotes spectrin binding to actin (for a review, see ref *10*). However, a puzzle is that several previous studies have reported that the pointed but *not* the barbed ends of the erythrocyte actin filaments are capped (*11*, *12*; for a review, see ref *5*), while other studies have reported that both ends of the erythrocyte actin filaments are uncapped (*13*, *14*).

Interestingly, both tropomodulin and adducin are unusual in comparison to other previously identfied actin filament capping proteins. For example, tropomodulin requires tropomyosin for high-affinity capping of actin filament pointed ends ($K_{cap} \sim 1$ nM) and is a relatively weak capping protein for pure actin filaments ($K_{cap} \sim 0.1-0.4$ μM) (*7*). This tropomyosin requirement may explain the presence of uncapped pointed ends in erythrocyte membranes isolated under conditions that result in tropomyosin dissociation from the membrane skeleton (e.g., *13*, *14*; for more discussion see ref *5*). Adducin is also a relatively weak barbed end capping protein with a K_{cap} of only ~100 nM (*9*), in contrast to barbed end capping proteins such as gelsolin or capZ (also referred to as "capping protein"), which have picomolar and nanomolar affinities for barbed filament ends, respectively (*1*, *2*). Furthermore, adducin is also unlike typical barbed end capping proteins in that it can bind along the sides of actin filaments and bundle them; this side-binding activity is required for its barbed end capping activity (*9*). A further difference from other barbed end capping proteins is that adducin binds calmodulin (*15*); calcium and calmodulin down regulate adducin's barbed end capping activity (*9*) as well as adducin's ability to bundle actin filaments and to promote spectrin−actin interactions (*16*, *17*).

In this study, we report that mature erythrocytes also contain abundant quantities of a nonmuscle isoform ($\alpha_1\beta_2$) of capZ, which has recently emerged as a major capping protein in platelets, neutrophils, and other nonmuscle cells (*2*, *18*−*21*). Surprisingly, in spite of the high affinity of erythrocyte capZ (EcapZ)[1] for actin filament barbed ends ($K_{cap} \sim 1-5$ nM), the EcapZ is all in the cytosol and is not associated with the membrane skeleton. Furthermore, purified EcapZ is unable to rebind to membranes that contain their full complement of adducin, while EcapZ does rebind to all the actin filament barbed ends in adducin-depleted membranes, suggesting that adducin may indeed cap the barbed ends of the erythrocyte actin filaments in intact erythrocytes. Previous reports that the barbed filament ends in ghosts were not capped (*11*−*14*, *22*) can presumably be explained by the use of adducin-depleted membranes in these experiments. The inability of the higher affinity capper, EcapZ, to compete with the apparently lower affinity capper, adducin, for the barbed ends of the erythrocyte actin filaments is likely to be due to unidentified factors in the erythrocyte membrane skeleton that increase the affinity of adducin for actin filament barbed ends. We speculate that the barbed ends of the erythrocyte actin filaments might represent a specialized class of membrane-associated actin filaments that are capped by adducin rather than by capZ or gelsolin.

EXPERIMENTAL PROCEDURES

Antibodies. Drs. Dorothy Schafer and John Cooper (Washington University School of Medicine, St. Louis, MO) generously provided us with a goat polyclonal antibody to chicken skeletal muscle capZ that recognized all α and β subunit isoforms, as well as a monoclonal antibody specific for the β_2 subunit present in non-muscle isoforms of capZ (*23*). We also obtained this monoclonal antibody (mAb3F.2) from the Hybridoma Bank at the University of Iowa. Rabbit antibodies to erythrocyte tropomodulin and tropomyosin were as described (*24*, *25*). Rabbit antibodies to the α subunit of human erythrocyte adducin were a gift from Dr. Vann Bennett, Howard Hughes Medical Institute, Durham, NC, and a monoclonal antibody to actin (C4) was a gift from Dr. John Lessard, University of Cincinnati, Cincinnati, OH.

Determination of Relative Amounts of Erythrocyte CapZ in Membranes and Cytosol. Small quantities of human erythrocytes were isolated from 10−20 mL of freshly drawn blood by sedimentation at 1*g* through four volumes of 0.75% (w/v) dextran T-500 (Pharmacia) at 4 °C (*26*) in phosphate-buffered saline (PBS) (10 mM sodium phosphate pH 7.5, and 150 mM NaCl). The cells were then washed three times in 10 volumes of PBS by centrifugation for 5 min at 1500*g* (4 °C), resuspended to a 20% suspension (v/v) with respect to the packed cell pellet, and stored on ice for up to 5 days. Before hemolysis, suitable amounts of washed cells were first sedimented at 1500*g* and the supernatant was aspirated off to prepare a packed cell pellet. In experiments to determine the relative amounts of EcapZ or other components in the cytosol or on the membrane (e.g., Figure 1), packed cells were lysed by addition of 10 volumes of ice-cold hypotonic lysis buffer (7.5 mM sodium phosphate, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT) (*27*) or by addition of 10 volumes of ice-cold saponin lysis buffer with physiological salts [0.1 M KCl, 2 mM $MgCl_2$, 20 mM Hepes, pH 7.3, 1 mM EGTA , 1 mM DTT, and 2.5% (w/v) saponin]. The membranes were pelleted for 15 min at top speed (approximately 14000*g*) in an Eppendorf microcentrifuge (4 °C) and then washed three times with 10 volumes of the same buffer to remove cytosol.

Membranes were resuspended to $^1/_5$ the original lysis volume in lysis buffer and equivalent amounts of packed cells, cytosol, or membranes (corresponding to 2.5 μL of packed cell-equivalents) were electrophoresed on SDS−7.5−15% linear gradient polyacrylamide gels containing 4 M urea with a 5% stacking gel containing 2 M urea (*28*, *29*). It was necessary to include urea due to the high concentrations

[1] Abbreviations: EcapZ, erythrocyte capZ; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; 5P8 ghosts, erythrocyte membranes prepared by lysis in 5 mM sodium phosphate, pH 8.0; Mg^{2+} ghosts, erythrocyte membranes prepared by lysis in 5P8 buffer containing 2 mM $MgCl_2$; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, D-*myo*-inositol 1,4,5-trisphosphate.

of hemoglobin present in the whole cells and cytosol samples. Gels were either stained for Coomassie blue R250 or transferred to nitrocellulose (0.2 μm; Schleicher and Schuell, Inc., Keene, NH) for 4 h at 100 V (cooled to 15 °C) as described (*30*), and then blots were stained with 0.2% ponceau S in 3% trichloroacetic acid to detect the erythrocyte membrane proteins. Strips at the appropriate molecular weight regions containing either the 32 kDa β_2 subunit of EcapZ or various membrane skeleton proteins were excised horizontally across all lanes of the blot, heated for 1 h in PBS at 65 °C (*29*) and nonspecific sites were blocked by incubation in 4% bovine serum for 2−4 h at room temperature. The β_2 subunit of EcapZ was detected by labeling overnight at 4 °C with a 1:40 dilution of the culture supernatant of mouse monoclonal antibody mAb3F.2 to the capZ β_2 subunit (equivalent to 10 μg/mL antibody), followed by labeling for 2−4 h at 4 °C in a 1:1000 dilution of rabbit anti-mouse IgG/IgM (Pierce), and finally with 10^6 cpm/mL ^{125}I-protein A (*31*). Other membrane skeleton proteins (actin, tropomodulin, tropomyosin, and adducin) were detected using primary antibodies as described above. Antibody incubation solutions and washing of blots between antibody incubations were as described (*24*). Labeled polypeptides were detected by exposure (typically 1−3 h) to X-ray film at −80 °C (X-OMAT AR film; Eastman Kodak Co., Rochester, NY).

Purification of EcapZ. EcapZ was purified from the cytosol of hypotonically lysed erythrocytes by a modification of the method of Casella and Cooper (*32*). The erythrocytes from six units of freshly drawn blood (General Clinical Research Center, Scripps Research Institute) were isolated from other blood cells and plasma by addition of four volumes of 0.2% gelatin at 25 °C in phosphate-buffered saline (PBS-EDTA) (7.5 mM sodium phosphate, pH 7.5, 150 mM NaCl, and 1 mM EDTA) followed by sedimentation at 1*g*. Cells were washed three times in 10 volumes of PBS-EDTA by repeated centrifugation at 1500*g*, 4 °C for 5 min followed by resuspension in PBS-EDTA, and then lysed hypotonically with 16 L of ice-cold lysis buffer (7.5 mM $NaHPO_4$, pH 7.5, 1 mM EDTA, and 2 mM DTT). Proteins in the lysate were absorbed to 300 mL of DEAE-Sephacel resin (Pharmacia) with stirring for 30 min at 4 °C. The DEAE resin was allowed to settle and, following decanting of the lysate, the resin was packed into a column (6 × 20 cm) and washed with 3 L of buffer T (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.05% 2-mercaptoethanol, and 0.0025% NaN_3) containing 50 mM NaCl. The EcapZ was eluted from the column by buffer T containing 150 mM NaCl, dialyzed against a large volume of buffer T to remove salt, and reapplied to a 30 × 2.5 cm DEAE Fast-Flow column (Pharmacia). The EcapZ was eluted with a linear gradient from 50 to 250 mM NaCl in buffer T. Fractions containing EcapZ were identified by western blotting with mAb3F.2 (see below), and then pooled and subjected to ammonium sulfate fractionation. The 50−70% pellet was resuspended in buffer T and loaded onto a 100 × 1.6 cm Superose 6 gel-filtration column and eluted with buffer T at a flow rate of 0.5 mL/min by FPLC. The major peak was pooled and dialyzed into 20 mM MES (pH 6.0) before being loaded onto a Pharmacia FPLC Mono S column (8 mL). The EcapZ was eluted using a linear gradient from 0 to 300 mM NaCl, where it eluted between 40 and 80 mM NaCl. Fractions containing EcapZ were detected by western blotting after electrophoresis on SDS−12% polyacrylamide gels using a Bio-Rad mini gel apparatus (*28*). To speed up detection of EcapZ, blots were blocked with BSA for only 1 h and also labeled for 1 h at room temperature with mAb3F2 to the β_2 subunit of capZ, then for 1 h at room temperature with rabbit anti-mouse IgG/IgM (1:1000; Pierce, Rockford, IL), and finally for 1 h at room temperature with ^{125}I-protein A, before detection of labeled β_2 subunit by exposure to X-ray film. Peak fractions were pooled, dialyzed into buffer T at a pH of 6.0, and subjected to a final purification on a Pharmacia Mono Q column (1 mL) with a 0−400 mM NaCl linear gradient. Under these conditions the EcapZ eluted between 190 and 210 mM NaCl and was essentially homogeneous. The EcapZ concentration was quantitated using an extinction coefficient of $\epsilon_{280}^{1mg/mL} = 1.25$ (*33*). Characterization of purified EcapZ by two-dimensional gel electrophoresis was as described by O'Farrell (*34*) and as modified by Granger et al. (*35*), using 5% polyacrylamide isoelectric focusing tube gels in the first dimension and SDS−12% polyacrylamide gels (pH 8.6) with a 5% stacking gel (pH 6.8) (*24*) in the second dimension.

To prepare crude cytosolic EcapZ, cytosol was collected after hypotonic lysis and centrifugation of membranes and concentrated by batchwise adsorption to a volume of DEAE-cellulose (Whatman DE53) equivalent to approximately the original packed cell volume. After the DEAE-cellulose was packed into a column, and washed extensively with hemolysis buffer to remove hemoglobin, bound cytosolic proteins (including EcapZ) were eluted with 0.05 M NaCl in hemolysis buffer followed by a second elution with 0.5 M NaCl in hemolysis buffer. Western blotting of cytosol and the various column washes demonstrated that all of the EcapZ in the cytosol was bound to DEAE-cellulose under these conditions and was eluted by 0.5 M NaCl along with the majority of the other cytosolic proteins (data not shown). The 0.05 and 0.5 M NaCl column washes were dialyzed into a large volume of EcapZ binding buffer in preparation for the binding assays (see below).

Actin Purification, Pyrene Labeling, and Polymerization Assays. Actin was purified from an acetone powder of rabbit skeletal muscle as described by Pardee and Spudich (*36*). To remove actin nuclei and other nucleating agents, the actin was passed over a 100 × 1.6 cm Superose 6 (Pharmacia) gel-filtration column by FPLC at 0.5 mL/min in buffer A. The fractions containing G-actin were polymerized by the addition of salts and tested by falling ball viscometry (*37*, *38*); those fractions exhibiting the highest viscosity (indicating the least contamination by nuclei) were pooled and the protein concentration was determined by absorbance at 290 nm, using an extinction coefficient of 26 600 M^{-1} cm^{-1}. The monomeric actin was stored on ice for up to 1 week. *N*-(1-Pyrene)iodoacetamide (P-29) was obtained from Molecular Probes, Inc. (Eugene, OR), and actin was labeled by pyrene using the method of Kouyama and Mihashi (*39*) with the modifications of Weber et al. (*40*). Pyrene-labeled actin was separated from unincorporated label by gel filtration over a 30 × 0.5 cm Superose 6 gel-filtration column by FPLC (Pharmacia). The pyrene-actin concentration was determined using an extinction coefficient of 22 000 M^{-1} cm^{-1} at 344 nm for the pyrene label and a correction factor for the actin concentration of $[Abs_{290} - (0.127Abs_{344})]/26\,600$. Pyrene-actin was stored on ice for up to 1 week.

Actin elongation was assayed as previously described (*9*) using the method of Casella et al. (*41*) in which polymerization of G-actin (5% pyrene labeled) was seeded using a crude low ionic strength extract of erythrocyte membranes containing spectrin−actin complexes with free barbed ends (*42*). Actin nucleation was assayed by the method of Kilimann and Isenberg (*43*). Briefly, 5 μM G-actin (5% pyrenyl actin) was first mixed with 100 nM EcapZ in buffer A, and actin polymerization was initiated by increasing the KCl concentration to 20 mM and the Mg^{2+} concentration to 1 mM. For assays in the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2), PIP_2 was added to the reaction mixture prior to the initiation of polymerization by salts. PIP_2 was obtained from Sigma (P9763) and was resuspended in 10 mM Tris-HCl (pH 8.0) by sonication for 2 min with a Microson sonicator before rapid freezing in liquid nitrogen. The aliquots were stored at −70 °C until used, thawed once, and sonicated in a sonicating water bath for 10 min prior to use.

EcapZ Binding Assay to Erythrocyte Membranes. To prepare membranes for EcapZ binding assays (e.g., Figures 6−9 , Table 1) packed cells prepared as described above were hemolysed in 30 volumes of ice-cold lysis buffer [5 mM sodium phosphate, pH 8.0, containing 1 mM dithiothreitol and 40 μg/mL phenylmethanesulfonyl fluoride (5P8 lysis buffer; *44*)], with or without 2 mM $MgCl_2$. It was critical to squirt the lysis buffer directly and rapidly onto the packed cell pellet while vortexing the cell pellet on a vortex mixer. After incubation of the hemolysate for 10 min on ice, membranes were collected by centrifugation at 40000*g* for 10 min (4 °C) and then washed 3 more times in 30 volumes of ice-cold lysis buffer, resuspended to the original volume of the 20% (v/v) cell suspension used to prepare the membranes, and stored on ice until used for the binding assays. Aliquots (50 μL) of packed membranes were prepared for binding assays by centrifugation of 250 μL of the washed membrane suspensions at top speed in an Eppendorf microcentrifuge for 10 min (4 °C). After aspiration of the supernatant, the packed membrane pellets were resuspended in 4 volumes (200 μL) of ice-cold EcapZ binding buffer (20 mM Hepes, pH 7.3, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, and 1 mM DTT) and purified EcapZ was added to a final concentration of 100 nM. Inclusion of Triton was necessary to prevent nonspecific sticking of EcapZ to the walls of the microfuge tubes. After incubation for 20−30 min at 0 °C, samples were underlaid with 200 μL of the same buffer containing 10% sucrose and centrifuged for 10 min at top speed in a microfuge (4 °C) to separate membrane-associated EcapZ from unbound EcapZ in the supernatant. After aspiration of the supernatant and the sucrose shelf, pellets were solublized in 200 μL of SDS sample buffer (*28*) and boiled for 3 min. Unless otherwise indicated, 60 μL amounts were electrophoresed on SDS−7.5−15% linear gradient polyacrylamide SDS (pH 8.6) for staining with Coomassie brilliant blue R250 (*24*), and 5 μL amounts (containing amounts of membranes corresponding to 1.25 μL of packed cells) were electrophoresed on SDS−10% polyacrylamide gels (pH 9.1) with a 5% stacker (pH 6.8) (*24*, *28*) in a Hoefer shortie gel apparatus for western blotting with mAb3F.2 to detect the β_2 subunit of EcapZ, as described above for determination of relative amounts of EcapZ in membranes and cytosol. Note that *both* the primary *and* secondary antibody incubations for these blots must be performed at 4 °C to achieve linear standard curves for EcapZ under the conditions of our assay. Labeled β_2 subunit was visualized by exposure to X-ray film at −80 °C (exposures were typically 3−4 h), and the portion of nitrocellulose corresponding to the β_2 subunit was excised from the blot and counted in a γ counter. In some experiments, the actual nanograms of EcapZ in each sample was determined with respect to a standard curve of purified EcapZ from 5−50 ng, electrophoresed in adjacent lanes on the same gel. Coelectrophoresis of purified EcapZ and isolated membranes (which contain negligible amounts of EcapZ; see Figures 1 and 6−9**)** demonstrated that these amounts of membranes did not interfere with the detection of EcapZ by this western blotting assay.

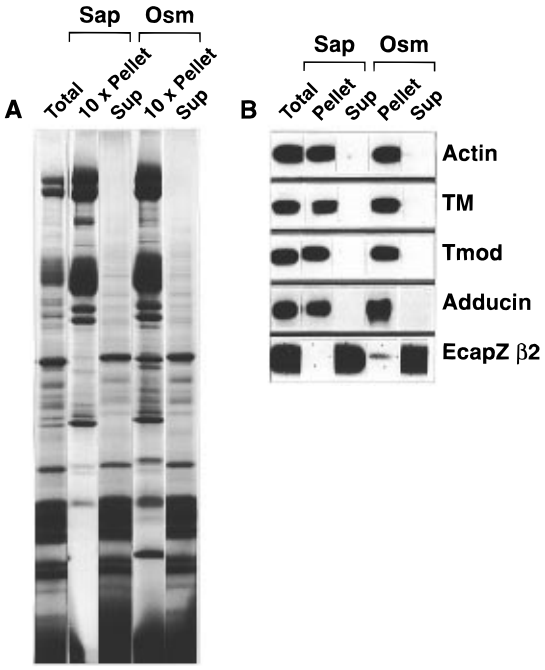

FIGURE 1: Comparison of amounts of EcapZ associated with membranes or in the cytosol after hypotonic or saponin hemolysis of human erythrocytes. (A) Coomassie blue stained gel and (B) Western blot of whole erythrocytes (Total), cytosol (Sup) and membranes (Pellet) prepared by saponin (Sap) or hypotonic lysis in the presence of magnesium (Osm) as described in Experimental Procedures. The indicated proteins on the Western blot were identified with specific antibodies to actin, tropomyosin (TM), tropomodulin (Tmod), adducin, and the β_2 subunit of EcapZ, followed by the appropriate secondary antibodies and ^{125}I-protein A as described in Experimental Procedures. Equivalent proportions of total erythrocytes, cytosol, and membranes were loaded for the western blots (B), while 10 times as much of the membrane pellet (10× Pellet) with respect to the total erythrocytes and cytosol was loaded for Coomassie blue staining (A).

RESULTS

Erythrocyte CapZ Is Present Exclusively in the Cytosol. A monoclonal antibody specific for the β_2 subunit of the capZ (also referred to as "capping protein") recognizes a polypeptide of the expected size (32 kDa) in western blots of whole human erythrocytes (Figure 1). Unlike in platelets (*19*, *20*) or striated muscle (*45*), where a substantial portion of capZ is associated with the actin filaments in the cytoskeleton or with the thin filaments, respectively, all of the erythrocyte capZ (EcapZ) is in the cytosol when cells are lysed either by hypotonic lysis in the presence of

magnesium or by saponin lysis in physiological salt (Figure 1B). Western blotting with a polyclonal antibody that recognizes both α and β capZ subunits demonstrates that the α subunit follows the β subunit and is also not associated with the membrane skeleton (data not shown). Coomassie blue staining or western blotting for other membrane skeleton proteins demonstrates that, in contrast to EcapZ, all of the erythrocyte actin and the other actin-binding proteins, including spectrin, band 4.1, tropomyosin, tropomodulin, and adducin, remain associated with the membrane and none of these proteins are in the cytosol under these conditions (Figure 1). Although a trace amount of EcapZ is detected in the membrane pellet after hypotonic lysis in the presence of magnesium, this is most likely due to nonspecific trapping of protein in resealed ghosts, since this is not observed after detergent lysis with saponin (Figure 1B). Inclusion of magnesium in hypotonic lysis buffers is well-established to promote membrane resealing; for example, ~1% of cytosolic hemoglobin is also trapped in ghosts prepared under these conditions (Figure 1A) (*44*). We were unable to find any hemolysis conditions that resulted in association of EcapZ with the membrane skeleton. For example, variation of calcium, magnesium, and salt concentrations during hypotonic or detergent (Triton X-100, saponin) hemolysis all had no effect on the cytosolic localization of the EcapZ (data not shown).

EcapZ Is a Heterodimer with a Subunit Composition of $\alpha_1\beta_2$. The absence of EcapZ in the membrane skeleton could be due to a defect in the EcapZ protein resulting in an inability of EcapZ to bind to actin filament barbed ends. To ascertain whether EcapZ was a normal αβ heterodimer and whether it was functionally active, we purified it from the cytosol of human erythrocytes, utilizing a series of different chromatographic columns and ammonium sulfate fractionation steps based on a purification protocol for capZ from skeletal muscle (Figure 2) (*32*). Purified EcapZ contains equal amounts of 36 and 32 kDa polypeptides by Coomassie blue staining, as expected for a capZ heterodimer (Figure 2, last lane, upper panel). Western blotting a duplicate gel with a polyclonal antibody prepared against chicken skeletal muscle capZ confirms that the 36 and 32 kDa polypeptides copurify and correspond to the α and β subunits of EcapZ (Figure 2, lower panel). In addition, sedimentation of purified EcapZ or whole unfractionated cytosol containing EcapZ on 5−20% sucrose density gradients followed by western blotting for EcapZ reveals an $S_{20,w}$ value for EcapZ of 4.8 (data not shown), similar to that reported for capZ heterodimers purified from other sources (*41*, *46*). The purity of EcapZ was in excess of 90% as determined by densitometry (Figure 2, last lane). On the basis of the yield of purified protein from six units of blood, and assuming a recovery of 10%, we estimate that the concentration of EcapZ in the cytosol of erythrocytes is about 200 nM,[2] somewhat lower than capZ concentrations in other nonmuscle cells: 1−2 μM in neutrophils (*18*) and 2−5 μM in platelets (*19*, *20*).

We characterized EcapZ by isoelectric focusing followed by SDS−polyacrylamide gel electrophoresis in the second dimension, first to ascertain the subunit isoform composition

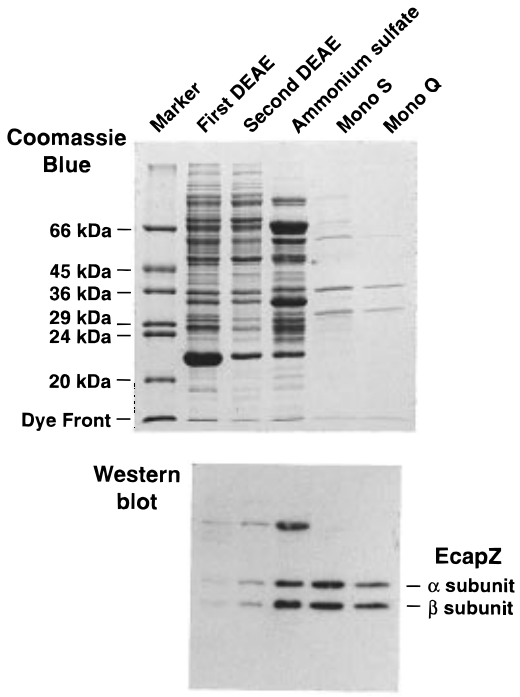

FIGURE 2: Purification of EcapZ from erythrocyte cytosol. EcapZ was purified from the cytosol of human erythrocytes using ion-exchange and gel-filtration chromatography and ammonium sulfate fractionation as described in Experimental Procedures. The relative purity of samples taken throughout the purification was ascertained by Coomassie blue staining after electrophoresis on SDS−12% polyacrylamide gels (Coomassie blue). The stages in the purification are indicated as follows: first DEAE, the 300 mL DEAE column; second DEAE, the 30 × 2.5 cm DEAE column; ammonium sulfate, the 50−70% ammonium sulfate cut; Mono S, the pooled fractions from the Mono S column; and Mono Q, the final pooled fractions from the Mono Q column. The identity of the purified protein as capZ was revealed by transferring an identical gel to nitrocellulose and labeling with a goat polyclonal antibody to chicken skeletal muscle capZ (Western blot) as described in Experimental Procedures. The molecular weight of standard markers and the position of the α and β capZ subunits are indicated.

and second to detect any posttranslational modifications that could potentially explain its absence from the erythrocyte membrane skeleton. Figure 3A illustrates diagrammatically the pattern of spots for the α and β subunit isoforms observed in purified EcapZ, and Figure 3B is a Coomassie blue stained two-dimensional gel of purified human EcapZ, with the spots labeled according to Schafer et al. (*23*). This gel indicates that the major α isoform in erythrocytes is the α_1 subunit, with a small amount of a spot in the α_1' subunit position. A very small amount of α_2 is also detected, although the amount is estimated to be about 100 times less than the amount of the α_1 subunit. Western blotting of the two-dimensional gel with a polyclonal antibody to chicken skeletal muscle capZ that recognizes all capping protein α and β isoforms (*23*) confirms that the major 36 and 32 kDa Coomassie blue stained spots correspond to the α_1 and β_2 subunits of EcapZ (Figure 3C). No β_1 isoform is present in erythrocytes. Furthermore, blotting with a monoclonal antibody specific for the β_2 subunit of capZ demonstrates that the spots at 32 kDa correspond to the β_2 and the β_2' spots (Figure 3D) (*23*). Western blots of two-dimensional gels of whole erythrocyte cytosol gave the same two spot patterns with the polyclonal antibody and the β_2 isoform-specific monoclonal antibody, indicating that a posttranslational modification was not lost during purification of EcapZ (data not shown). We conclude

[2] It was not possible to measure directly the amount of EcapZ in erythrocyte cytosol by a western blotting assay due to interference by the extremely high concentrations of hemoglobin in the cytosol.

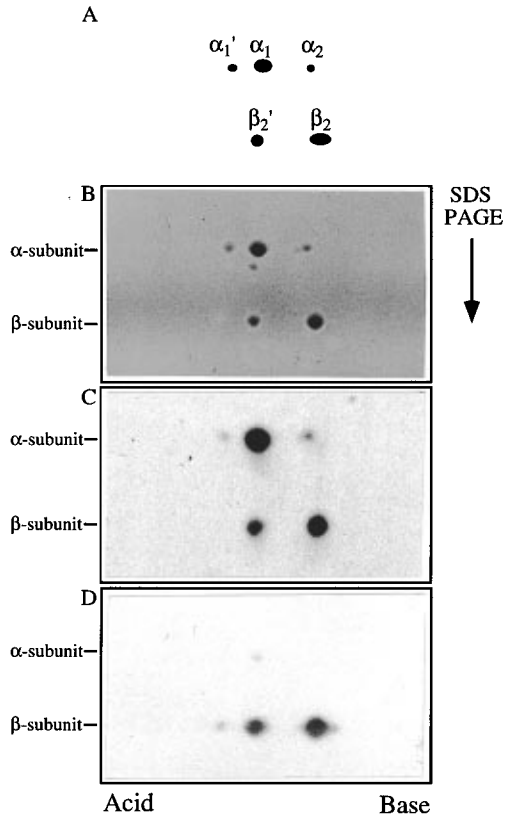

FIGURE 3: Two-dimensional gel electrophoresis of purified EcapZ. (A) Diagram indicating the positions of α and β subunits of EcapZ according to Schafer et al. (*23*). (B) Coomassie blue stained gel of purified EcapZ (3 μg). (C, D) Western blots of purified EcapZ detected by labeling with (C) a polyclonal anti-chicken skeletal muscle capZ antibody that recognizes both the capZ α and β subunits or (D) a monoclonal antibody specific for the β_2 subunit of capZ. The position of the acidic and basic ends of the first dimension isoelectric focusing gel and the direction of SDS−polyacrylamide gel electrophoresis in the second dimension are indicated.

that the isoform specificity of human EcapZ conforms to $\alpha_1\beta_2$ and shows no indication of posttranslational modification.

Purified EcapZ Caps Actin Filament Barbed Ends in Vitro. We showed previously that purified EcapZ greatly reduced the depolymerization rate of F-actin that was diluted below its critical concentration (Figure 2 in ref *9*), and also that polymerization of actin in the presence of increasing amounts of EcapZ resulted in an increase in the actin critical concentration, as expected for a protein that caps actin filament barbed ends without sequestering actin monomers (Figure 3 in ref *9*). To determine quantitatively whether the actin capping activity of purified EcapZ was similar to that of other capZ isoforms, we examined its ability to inhibit actin polymerization nucleated by isolated spectrin−actin oligomeric complexes containing free barbed ends (*7*, *41*, *42*). Figure 4A shows that increasing concentrations of EcapZ dramatically inhibit the initial polymerization rate of actin from spectrin−actin seeds, indicating that EcapZ caps their barbed ends. The EcapZ concentration that produces 50% inhibition of the initial polymerization rate is taken as a measure of the K_{cap}; in this experiment the K_{cap}= 5 nM (Figure 4B). Different preparations of EcapZ gave various values for the K_{cap} from 1 to 5 nM. These values are similar to the actin capping activity of capZ isoforms isolated from muscle ($\alpha_1\beta_1$ or $\alpha_2\beta_1$) or nonmuscle ($\alpha_1\beta_2$ or $\alpha_2\beta_2$) cells and tissues, respectively (*33*, *41*, *47*, *48*). In addition, we also observed that the K_{cap} of EcapZ for capping β-actin purified from human erythrocytes was similar to the K_{cap} of EcapZ for α-actin purified from rabbit skeletal muscle (data not shown), as also observed for $\alpha_1\beta_2$ or $\alpha_1\beta_1$ capZ isoforms purified from other tissues by Schafer et al. (*47*). Since capZ isoforms have the ability to nucleate actin polymerization, we also examined EcapZ for nucleating activity using the method of Kilimann and Isenberg (*43*). At a concentration of 100 nM, EcapZ abolished the lag phase in the actin polymerization curve, indicating that EcapZ is capable of nucleating actin polymerization (Figure 4C).

The capping activities of both muscle ($\alpha\beta_1$) and nonmuscle ($\alpha\beta_2$) isoforms of capZ are inhibited by PIP_2 (*47*, *49*, *50*). To ascertain whether PIP_2 also inhibits the activity of EcapZ, we determined the effect of PIP_2 on the percent capping at three different concentrations of EcapZ. Figure 5A shows that PIP_2 does inhibit the ability of EcapZ to cap actin filament barbed ends and that proportionately higher concentrations of PIP_2 are required to reduce EcapZ's capping activity as the concentration of EcapZ is increased. Indeed, a linear relationship is observed when the EcapZ concentrations are plotted versus the PIP_2 concentrations at which 50% capping was observed (Figure 5B). The linearity of this relationship is consistent with the idea that PIP_2 inhibits EcapZ activity by binding to and sequestering EcapZ, as previously proposed for other capZ isoforms (*47*, *50*, *51*). At a PIP_2 concentration of 10 μM the apparent K_{cap} of EcapZ is increased to 120 nM, and at a PIP_2 concentration of 50 μM the apparent K_{cap} of EcapZ is extrapolated to be about 575 nM. We conclude that the barbed end capping activity of purified EcapZ is not significantly different from any other previously characterized capping protein isoforms and thus a defect in EcapZ cannot account for its absence in erythrocyte membranes.

Cytosol Does Not Inhibit Binding of EcapZ to Actin Filament Barbed Ends. It is possible that the absence of endogenous EcapZ from isolated erythrocyte membranes might be explained by a cytosolic inhibitory factor. Due to optical intereference by the high concentrations of hemoglobin in erythrocyte cytosol, it was not possible to test directly the effect of cytosol on the capping activity of EcapZ using the pyrene-actin polymerization assay. Therefore, we compared the ability of endogenous, cytosolic EcapZ and purified EcapZ to bind to actin filament barbed ends using the 5P8 ghost binding assay described below (see Figure 7 and Table 1). We obtained cytosol by hypotonic hemolysis and concentrated the cytosolic proteins (which include EcapZ) back to the original cell volume by adsorption to DEAE-cellulose followed by sequential elution with 0.05 and 0.5 M NaCl washes. This procedure also removed hemoglobin since hemoglobin does not stick to DEAE-cellulose. Western blotting and Coomassie blue staining showed that all of the cytosolic EcapZ was adsorbed to the DEAE column and was eluted in the 0.5 M NaCl wash along with the majority of the other cytosolic proteins (data not shown). Figure 6 shows that the cytosolic proteins present in either the 0.05 or the 0.5 M NaCl washes of the DEAE column do not reduce the amount of purified EcapZ binding to free actin filament barbed ends in 5P8 ghosts (Figure 6, compare lanes 3 and 4 with lanes 7 and 8 and 11 and 12). In addition, the cytosolic EcapZ that is present in the 0.5 M NaCl wash from the DEAE column itself binds to actin filament barbed ends

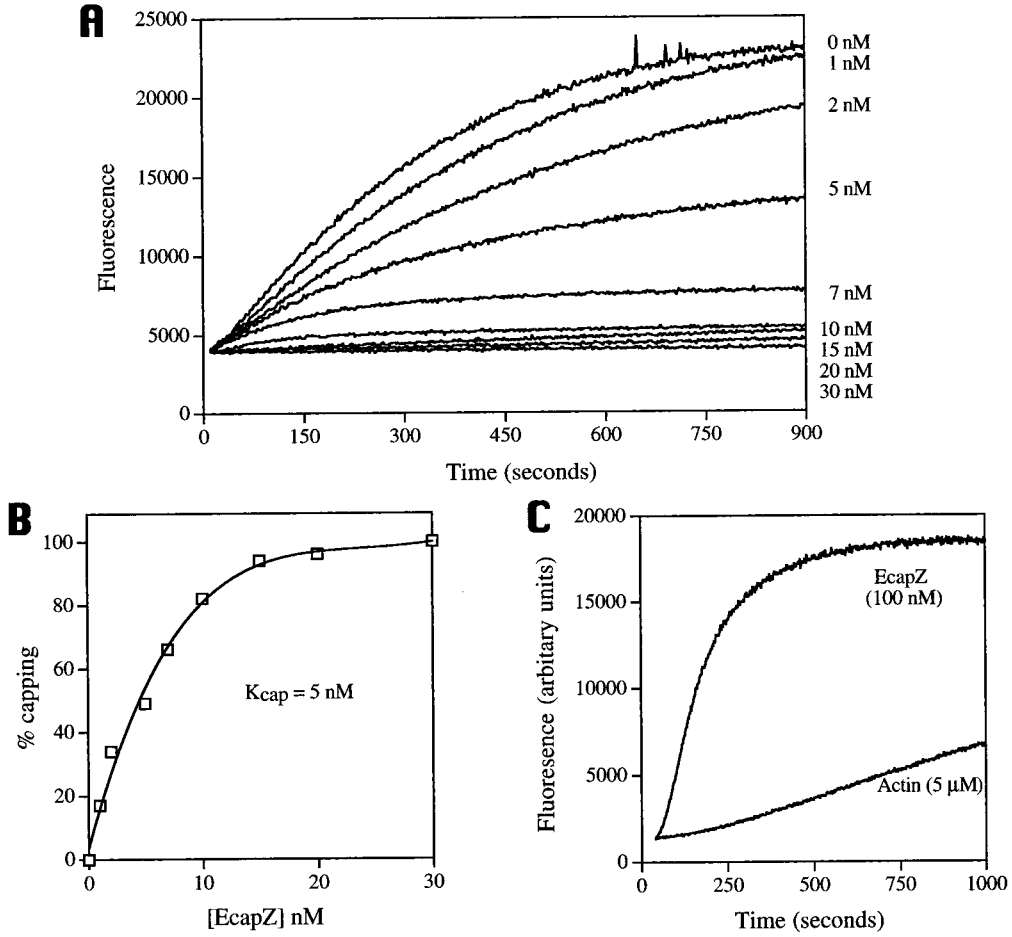

FIGURE 4: Characterization of the actin filament barbed end capping activity of EcapZ: Effect of EcapZ on the rate of polymerization from spectrin−actin nuclei. (A) Raw data. Elongation was inititated by the addition of spectrin-actin seeds and salts to a 5 μM G-actin solution (5% pyrenyl-actin) containing increasing concentrations of EcapZ as indicated. (B) Percent capping at different concentrations of EcapZ, plotted against increasing EcapZ concentration. The percent capping is calculated by dividing the initial rate of polymerization in the presence of EcapZ by the initial rate of polymerization in the absence of EcapZ; 100% capping is the complete cessation of actin polymerization. The concentration of EcapZ required to produce 50% inhibition of polymerization (5 nM) was taken as a measure of K_{cap}. (C) Demonstration of EcapZ's ability to nucleate actin polymerization. 5 μM G-actin (5% pyrene labeled) was mixed with 100 nM capping protein or buffer A and polymerization was initiated by adding KCl and Mg^{2+} as described in Experimental Procedures. Polymerization was followed by an increase in pyrene fluorescence and was plotted against time.

(Figure 6, lanes 9 and 10); the amount of bound cytosolic EcapZ is about the same as the amount of purified EcapZ bound at the saturating concentration (100 nM) of purified EcapZ used in the binding assay (Figure 6, compare lanes 9 and 10 with lanes 3 and 4). This demonstrates that the concentration of cytosolic EcapZ is not limiting and is at least 100 nM, in good agreement with our previous estimate of the cytosolic EcapZ concentration at 200 nM based on the yield from purification (see above).[2] These results indicate that cytosol does not inhibit binding of EcapZ to free actin filament barbed ends in membranes and thus a cytosolic inhibitory factor cannot account for the absence of EcapZ from isolated erythrocyte membranes.

Binding of EcapZ to Actin Filament Barbed Ends in 5P8 Ghosts but Not in Mg^{2+} Ghosts Suggests Barbed Ends Are Capped in Mg^{2+} Ghosts. Since EcapZ is fully functional in cytosol, the lack of association of EcapZ with the membrane skeleton could be due to prior capping of the barbed ends of the erythrocyte actin filaments, possibly by adducin. To test this, we studied binding of EcapZ to membranes prepared under a variety of conditions, using a cosedimentation assay followed by SDS−polyacrylamide gel electrophoresis and western blotting to detect EcapZ. We compared binding of EcapZ to membranes prepared by hypotonic hemolysis in 5 mM sodium phosphate buffer, pH 8.0 (5P8), or in 5P8 buffer containing 2 mM $MgCl_2$ (5P8 ghosts or Mg^{2+} ghosts, respectively). As described above, negligible amounts of endogenous EcapZ remained associated with the isolated membranes prepared under either condition (Figures 1B and 6−9). Membranes were incubated with a saturating concentration of EcapZ (100 nM) in a physiological salt buffer containing 0.1% Triton X-100 to permeabilize the membranes and to prevent nonspecific sticking of EcapZ, and then bound EcapZ was separated from unbound EcapZ by sedimentation through a sucrose shelf. Figure 7A shows that the amount of purified EcapZ cosedimenting with Mg^{2+} ghosts is barely above the amount of residual EcapZ present in the isolated membranes. Incubation of the Mg^{2+} ghosts with higher concentrations of EcapZ up to 200 nM does not result in any more EcapZ cosedimenting with the ghosts, indicating that the available binding sites have been saturated (data not shown). Quantitation of the amount of EcapZ bound to the Mg^{2+} ghosts shows that only one EcapZ heterodimer is present for every 165 actin subunits (Table 1). Assuming that the erythrocyte actin filaments are about 15 monomers long (*3*, *11*, *22*), this indicates that less than 1/10 of the actin filaments have a bound EcapZ molecule and implies that the barbed ends of most of the actin

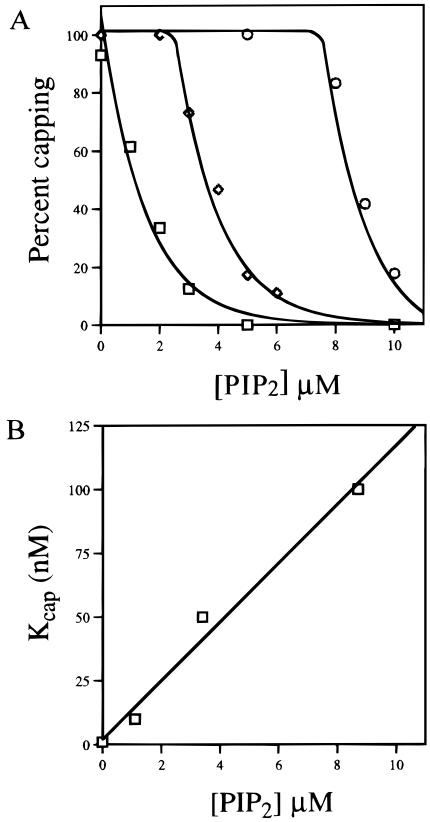

FIGURE 5: PIP_2 inhibits the capping activity of EcapZ. (A) Capping activity of (□) 10 nM, (◇) 50 nM, and (○) 100 nM EcapZ in the presence of increasing PIP_2 concentrations. By use of the exponential nonlinear least-squares fit feature of the Cricket Graph software package, a curve was fitted to the 10 nM EcapZ data. This same curve when displaced along the *x*-axis adequately describes the data for the other two concentrations of capping protein (50 and 100 nM). (B) The concentration of PIP_2 responsible for inhibiting EcapZ capping activity by 50% was taken as a measure of the K_{cap} of capping protein at these PIP_2 concentrations. These capping constants were plotted against PIP_2 concentration to determine the effect of PIP_2 on capping activity. The curves were fitted with the exponential option of the Cricket Graph software package for Macintosh, and the relationship between K_{cap} and [PIP_2] was determined by linear regression analysis.

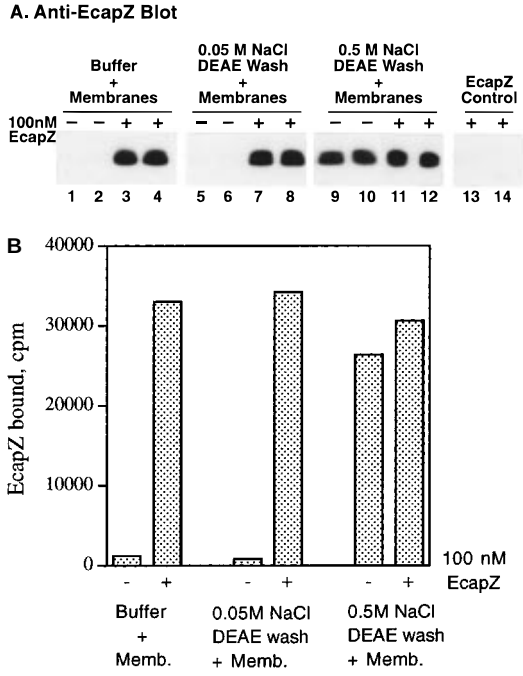

FIGURE 6: Effect of cytosol on binding EcapZ to actin filament barbed ends in 5P8 ghosts. (A) Anti-EcapZ Western blot of 5P8 ghosts incubated in the absence (−) or presence (+) of 100 nM purified EcapZ together with concentrated cytosolic proteins obtained from the 0.05 or 0.5 M NaCl wash of a DEAE-cellulose column to which total erythrocyte cytosol had been adsorbed (see Experimental Procedures). After separation from unbound EcapZ by sedimentation through a 10% sucrose shelf, the amount of EcapZ cosedimenting with membranes was determined by western blotting for the β_2 subunit. (B) Quantitation of the amount of EcapZ bound to membranes (EcapZ bound, counts per minute) in the experiment depicted in panel A. The counts per minute associated with the EcapZ-β_2 polypeptide in membranes were corrected by subtraction of the counts in the same region of the blot when EcapZ was sedimented in the absence of membranes (1600 cpm); this was similar to the background counts per minute in a similarly sized piece of nitrocellulose from a blank region of the blot (1200 cpm). Aliquots of membranes containing free actin filament barbed ends were prepared by hypotonic hemolysis in 5P8 buffer (see Figure 7 and Table 1) and preincubated with 4 volumes of the dialyzed, 0.05 or 0.5 M NaCl salt washes for 30 min at 0 °C in the presence of 0.1% Triton X-100. After addition of 100 nM purified EcapZ and incubation for an additional 30 min at 0 °C, membranes were sedimented through a 10% sucrose shelf to separate bound from unbound EcapZ and the amount of bound EcapZ was determined by Western blotting for the EcapZ-β_2 subunit as described. Note that cytosolic EcapZ is present in the 0.5 M NaCl wash of the DEAE-cellulose column and cosediments with membranes to the same extent as does purified EcapZ in the absence of cytosolic proteins.

filaments are already capped by another factor in Mg^{2+} ghosts. In some experiments, even smaller amounts of EcapZ were observed to bind to Mg^{++}ghosts (e.g., see Figure 9). Likewise, only small amounts of purified EcapZ were observed to cosediment with membranes prepared by saponin lysis in physiologial salt, suggesting that the actin filament barbed ends are also capped in membranes prepared this way (data not shown).

In contrast, substantial amounts of EcapZ do cosediment with 5P8 ghosts that are prepared by hypotonic hemolysis in the absence of magnesium (Figure 7B). Incubation of 5P8 ghosts with higher concentrations of EcapZ up to 200 nM does not result in more EcapZ cosedimenting with the ghosts, again suggesting that the available binding sites have been saturated (data not shown). Quantitation of the amount of EcapZ bound with respect to the amount of actin in the 5P8 ghosts reveals a ratio of 1 EcapZ heterodimer present per 16 actins (Table 1). This binding stoichiometry indicates that EcapZ is likely to be binding to the barbed ends of all of the short actin filaments in the membrane skeleton. In support of this interpretation, preincubation of 5P8 membranes with 1 μM cytochalasin D inhibited the amount of EcapZ cosedimenting with 5P8 ghosts by over 90% (data not shown). Thus, in agreement with previous studies, after hemolysis in 5P8 buffer, all the actin filament barbed ends appear to be uncapped and available for binding by EcapZ (*14*, *22*).

Ability of EcapZ To Bind to Free Barbed Ends in 5P8 Ghosts Is Likely Due to Loss of Adducin and Not to Decreases in PIP_2 or to Creation of New Ends by Filament Breakage. We tested several possibilities in an attempt to determine why EcapZ binds to actin filament barbed ends in 5P8 ghosts but not in Mg^{2+} ghosts. First, since PIP_2 can reduce the apparent affinity of purified EcapZ for actin filament barbed ends *in vitro* (Figure 5) and erythrocytes contain high concentrations of PIP_2 (~50 μM; see ref *52*), we reasoned that Mg^{2+} ghosts might contain more PIP_2 than 5P8 ghosts and that this might account for the low level of

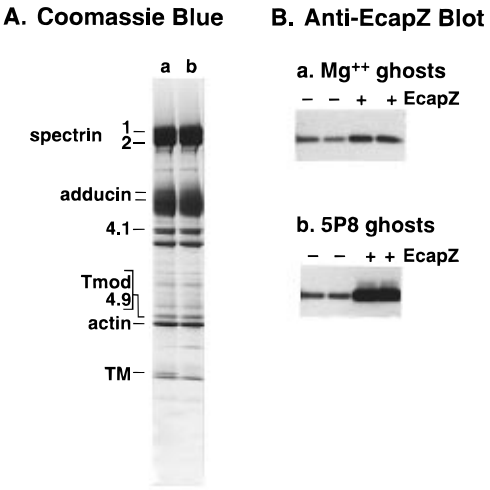

FIGURE 7: EcapZ binds to actin filament barbed ends in 5P8 ghosts but not in Mg^{2+} ghosts. (A) Coomassie blue stained SDS−polyacrylamide gel of erythrocyte membranes prepared by hypotonic hemolysis in (a) 5 mM sodium phosphate buffer, pH 8.0, with 2 mM $MgCl_2$ (Mg^{2+} ghosts) or (b) 5 mM sodium phosphate buffer, pH 8.0 (5P8 ghosts). (B) Anti-EcapZ Western blot of 5P8 or Mg^{2+} ghosts incubated in the absence (−) or presence (+) of 100 nM purified EcapZ and separated from unbound EcapZ by sedimentation through a 10% sucrose shelf. After solubilization of membrane pellets in SDS sample buffer, 80 μL samples (equivalent to 20 μL of packed membranes) were electrophoresed on 7.5−15% linear gradient SDS−polyacrylamide gels (pH 8.6) for Coomassie blue staining (A), while duplicate 40 μL samples (equivalent to 10 μL of packed membranes) were electrophoresed on SDS−10% polyacrylamide gels for western blotting for the EcapZ-β_2 subunit (B). In separate controls (not shown), we determined that the amount of EcapZ sedimenting in the absence of membranes was barely detectable above background labeling of the blots (also see Figure 6, lanes 13 and 14). The migration positions of erythrocyte membrane skeleton proteins are indicated on the Coomassie blue gel in panel A. Note that the α and β polypeptides of adducin are barely visible due to the band 3 protein, which migrates as a broad, diffuse band in this region of the gel.

EcapZ binding to the Mg^{2+} ghosts. However, when we quantitated the amount of PIP_2 in the two types of ghosts using an IP_3 competition binding assay (after alkaline hydrolysis of PIP_2 to IP_3) (*53*), we found that both types of ghosts actually had about the same amount of PIP_2 (∼20 μM, data not shown). Furthermore, the EcapZ binding assay is performed in 0.1% Triton X-100 and 2 mM $MgCl_2$ for both types of ghosts; Triton dissociates PIP_2 micelles and magnesium screens the phosphate head groups, reducing the efficacy of PIP_2 at sequestering capZ and thereby obviating PIP_2 inhibition of capZ binding to actin filament ends. Thus, PIP_2 in the membrane cannot account for the inability of EcapZ to bind to the actin filament barbed ends in Mg^{2+} ghosts.

Second, since tropomyosin has been reported to reduce the susceptibility of pure actin filaments to mechanical breakage (*54*, *55*), and tropomyosin is lost from membranes prepared by hypotonic hemolysis in the absence of magnesium (*27*) (also see Figure 9B), we reasoned that the absence of tropomyosin from 5P8 ghosts could potentially lead to increased filament breakage during hemolysis and washing, thus generating more free ends. We tested this by comparing EcapZ binding to ghosts prepared in 5P8 buffer containing 0, 10, or 80 μM phallacidin. Phallacidin is a water-soluble analogue of phalloidin, a bicyclic peptide that binds specifically and tightly to the subunits in the actin polymer and greatly enhances actin filament stability, reducing fragmentation as well as depolymerization (*56*). Inclusion of phallacidin in lysis buffers has no effect on the protein composition of isolated membranes as revealed by Coomassie blue staining (Figure 8, panel A). The amount of EcapZ binding to 5P8 ghosts prepared in 10 or 80 μM phallacidin is 80% and 65%, respectively, of the amount of EcapZ binding to 5P8 ghosts prepared in the absence of phallacidin (Figure 8, panel B). In contrast, the amount of EcapZ binding to Mg^{2+} ghosts in this experiment is only 6% of the amount of binding to 5P8 ghosts (Figure 8). This experiment indicates that filament breakage can account for only a relatively minor portion of the dramatic differences in EcapZ binding capacity (i.e., numbers of free barbed ends) of 5P8 ghosts as compared to Mg^{2+} ghosts.

Finally, we wondered whether, like tropomyosin, adducin might also be lost from membranes prepared by hypotonic hemolysis in the absence of magnesium. Indeed, Figure 9 shows that 5P8 ghosts contain only 40% of the amount of adducin present in the equivalent amount of Mg^{2+} ghosts (Figure 9C) and only 10% of the tropomyosin that is present in the Mg^{2+} ghosts (Figure 9B). In other experiments, the amount of adducin remaining associated with the membranes of 5P8 ghosts varied between 20% and 40% (data not shown). This contrasts with other membrane skeleton proteins such as spectrin, protein 4.1, protein 4.9, actin (Figure 9A), and tropomodulin (Figure 9D), which, as expected, are tightly associated with the membrane skeleton and present at the same levels in either 5P8 or Mg^{2+} ghosts. Furthermore, comparison of amounts of adducin, tropomyosin, and all of the membrane skeleton proteins in intact cells, cytosol, and membranes demonstrates that Mg^{2+} ghosts

Table 1: Quantitation of Free Actin Filament Barbed Ends in Isolated Membranes As Detected by Binding of EcapZ

ghosts	EcapZ bound[a] (cpm)	EcapZ bound[b] (ng)	mol of actin/mol of EcapZ[c]
Mg^{2+} ghosts	3422	6.0	165.0
5P8 ghosts	35 430	62.4	15.8

[a] Binding of purified EcapZ to membranes prepared by hemolysis in 5P8 buffer (5P8 ghosts) or 5P8 buffer containing 2 mM $MgCl_2$ (Mg^{2+} ghosts) was determined by a cosedimentation assay followed by SDS−polyacrylamide gel electrophoresis and western blotting for the β_2 subunit of EcapZ, as described in the caption to Figure 7 and in Experimental Procedures. The amount of purified EcapZ cosedimenting with membranes after incubation with 100 nM EcapZ is corrected by subtraction of the amount of residual EcapZ in isolated membranes (see Figure 7). This residual amount of EcapZ in 5P8 or Mg^{2+} ghosts is less than 10% of the amount of purified EcapZ that cosediments with the 5P8 ghosts. The amount of EcapZ sedimenting in the absence of membranes is negligible and is not detectable above the background counts per minute on the nitrocellulose blot (see Figure 6, lanes 13 and 14). Data are averages of duplicate samples from the experiment shown in Figure 7. [b] Nanograms of EcapZ determined from a standard curve of 5−50 ng of EcapZ electrophoresed in adjacent lanes on the same gel. [c] The moles of actin/mole of EcapZ bound was determined according to the following formula: moles of actin/moles of EcapZ = (nanograms of actin in membranes/nanograms of EcapZ bound)(MW EcapZ β_2 subunit/MW actin). The MW of actin was 42 000, and that of EcapZ β_2 subunit was 32 000. For quantitation of actin, rabbit skeletal muscle actin standards from 0.5 to 2.5 μg were electrophoresed on 7.5−15% linear gradient polyacrylamide−SDS gels (pH 8.6) (*24*) in parallel with duplicate 40 μL samples of solubilized membrane pellets (equivalent to 10 μL of packed membranes) from the binding assay shown in Figure 7. After being stained with Coomassie blue, actin bands were excised and the amount of protein was quantitated by elution with 0.5 mL of 25% (v/v) pyridine, as described (*63*). There was 1.3 μg of actin/10 μL of packed membranes for both 5P8 and Mg^{2+} ghosts.

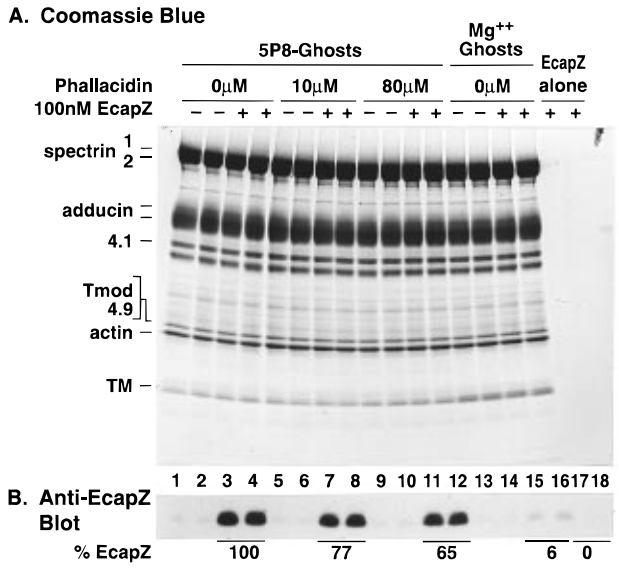

FIGURE 8: Effect of inclusion of phallacidin during hemolysis on binding of EcapZ to 5P8 ghosts. (A) Coomassie blue stained SDS−polyacrylamide gel and (B) Western blot of EcapZ bound to erythrocyte membranes prepared by hypotonic hemolysis in 5P8 buffer (5P8 ghosts) containing 0, 10, or 80 μM phallacidin or in 5P8 buffer containing 2 mM $MgCl_2$ (Mg^{2+} ghosts). In panel B the amount of EcapZ bound was determined as a percentage of the amount of EcapZ cosedimenting with 5P8 ghosts (% EcapZ). Membranes were prepared by hypotonic hemolysis in 5P8 buffer (lanes 1−4) containing 10 μM (lanes 5−8) or 80 μM (lanes 9−12) phallacidin (Sigma) or 2 mM $MgCl_2$ (lanes 13−16) as described in Experimental Procedures, with the exception that membrane pellets were washed twice only after the initial hemolysis and centrifugation step. Duplicate aliquots of membranes were incubated for 30 min at 0 °C in the presence (+) or absence (−) of 100 nM EcapZ (lanes 1−16), sedimented through a 10% sucrose shelf to separate bound from unbound EcapZ, and the amount of bound EcapZ was determined by western blotting for the EcapZ-β_2 subunit as described. Lanes 17 and 18 are a control with 100 nM EcapZ incubated and sedimented in the absence of membranes. The amounts of EcapZ-β_2 subunit cosedimenting with membranes (lanes 3 and 4, 7 and 8, 11 and 12, and 15 and 16) were corrected by subtraction of the amount of residual EcapZ-β_2 subunit associated with membranes incubated in the absence of EcapZ (lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14). The amount of EcapZ sedimenting in the absence of membranes was not above the background labeling of the blot (lanes 17 and 18). The positions of the erythrocyte membrane skeleton proteins are indicated as in Figure 7.

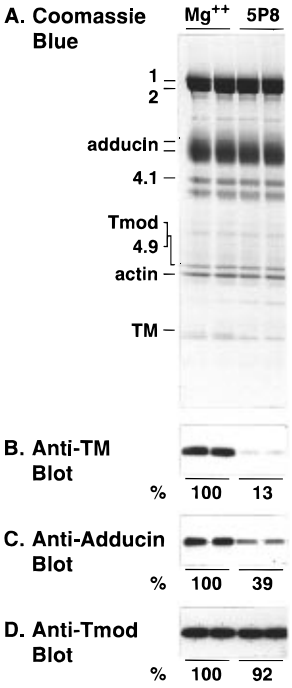

FIGURE 9: Adducin and tropomyosin are depleted from 5P8 ghosts in comparison to Mg^{2+} ghosts. (A) Coomassie blue stained gel and western blots of (B) tropomyosin (TM), (C) adducin, and (D) tropomodulin (Tmod) in ghosts prepared by hypotonic hemolysis in 5P8 buffer in the absence of magnesium (5P8) or ghosts prepared by hypotonic hemolysis in 5P8 buffer containing 2 mM $MgCl_2$ (Mg^{2+}). Membranes were prepared by hypotonic hemolysis as described in Experimental Procedures for the EcapZ binding assays, and equivalent amounts were electrophoresed on SDS−7.5−15% linear gradient polyacrylamide gels and then stained with Coomassie blue or transferred to nitrocellulose for western blotting with antibodies to tropomyosin, adducin, or tropomodulin. After exposure to X-ray film, pieces of nitrocellulose with the labeled polypeptides were excised and counted, in a γ counter and the counts per minute associated with each band were expressed as a percentage of the amount present in the Mg^{2+} ghosts. In separate experiments (e.g., see Figure 1), we determined that the amounts of each protein present in the Mg^{2+} ghosts were the same as those in intact cells. The positions of the erythrocyte membrane skeleton proteins are indicated as in Figure 7.

contain their full complement of these proteins and that none of these proteins are present in the cytosol (Figure 1). Taken together, our results are consistent with the idea that adducin caps the actin filament barbed ends in Mg^{2+} ghosts, preventing EcapZ from binding to them, while loss of adducin during lysis in 5P8 buffer leads to artifactual filament uncapping of most of the filaments, thus allowing EcapZ to bind to the newly freed barbed ends.

DISCUSSION

In this study, we show that human erythrocytes contain a nonmuscle isoform of the heterodimeric actin filament barbed end capping protein, capZ, based on western blotting with specific antibodies and characterization of the purified protein. The isoform composition of erythrocyte capping protein (EcapZ) is $\alpha_1\beta_2$, with no β_1 subunit, similar to other nonmuscle cells. Erythrocytes do not contain significant amounts of the α_2 isoform, unlike striated muscle, platelets, and other nonmuscle cells and tissues (*19*, *20*, *23*, *47*, *57*). Characterization of the effects of purified EcapZ on actin polymerization indicates that it is fully functional in capping actin filaments. It blocks elongation and depolymerization from the barbed ends of actin filaments with a K_{cap} of 1−5 nM and also nucleates actin polymerization, as described previously for capZ isolated from skeletal muscle (*41*, *33*) and a variety of nonmuscle cells and tissues (*43*, *46*−*48*; for a review see ref *2*).

EcapZ is exclusively located in the cytosol and is not associated with the actin filament barbed ends in the erythrocyte membrane skeleton under any hemolysis condition used. This contrasts with platelets, where some capZ is in the cytosol and some is associated with actin filament barbed ends in the Triton-insoluble cytoskeleton (*19*, *20*), or striated muscle, where all of the capZ is associated with the actin filament barbed ends at the Z disks (*45*, *58*). The absence of EcapZ from the actin filament barbed ends in erythrocyte membrane skeletons is not due to a defect in EcapZ, to a cytosolic inhibitory factor, or to insufficient concentrations of EcapZ in erythrocytes (cytosolic EcapZ concentration is estimated to be 100−200 nM). In addition, it is unlikely that EcapZ dissociates from the actin filament barbed ends during hemolysis and sedimentation of mem-

branes, since the slow off-rate constant reported for capZ predicts a ~30 min half-life for capped barbed ends (*47*).

Therefore, we propose that EcapZ is not associated with the erythrocyte membrane skeleton because the actin filament barbed ends are already capped by another factor. We suggest that this factor is adducin, a unique membrane-associated protein that has been recently shown to cap actin filament barbed ends *in vitro* (*9*). Evidence in support of this hypothesis is that purified EcapZ is unable to bind to actin filament barbed ends in Mg^{2+} ghosts that retain their full complement of adducin, but it can bind to actin filament barbed ends in 5P8 ghosts from which most of the adducin (or another factor) has been lost during preparation of membranes. Thus, we propose that the barbed ends of the short erythrocyte actin filaments are indeed capped *in situ* in intact cells and that dissociation of adducin from the membrane during membrane isolation in 5P8 buffer leads to artifactual filament uncapping at the barbed end. This can also account for the presence of free barbed ends in the oligomeric spectrin−actin complexes that we and others have used to seed actin polymerization, since these complexes are purified from ghosts made in the absence of magnesium (e.g., *7*, *9*, *41*, *42*). A model depicting the association of adducin with the barbed ends of the short erythrocyte actin filaments is presented in a recent review (*5*).

Our work raises the following dilemma: erythrocytes contain two actin filament barbed end capping proteins, one with a high affinity (EcapZ, $K_{cap} \sim 1-5$ nM) and one with a lower affinity (adducin, $K_{cap} \sim 100$ nM, ref *9*), but of the two, adducin is associated with the actin filaments in the membrane skeleton whereas EcapZ is not. How does adducin with the lower affinity for actin outcompete EcapZ that apparently has a greater than 20-fold higher affinity? We favor the hypothesis that additional cofactors in the membrane skeleton may increase the afffinity of adducin for the actin filament barbed ends. For example, adducin binds more tightly to spectrin−actin complexes than to pure actin filaments (*16*, *17*); thus interaction of adducin with spectrin−actin complexes in the membrane skeleton may increase its affinity for actin filament barbed ends. The actin filament barbed end capping activity of adducin may also be upregulated by specific phosphorylation or dephosphorylation reactions since phosphorylation of adducin by protein kinases A and C has complex effects on adducin's interactions with spectrin−actin complexes and with calmodulin (*59*). Although the actin capping activity of unphosphorylated adducin is downregulated by calcium−calmodulin (*9*), phosphorylation of adducin may potentially modulate this effect (*59*). Experiments are in progress to test these possibilities.

An alternative explanation based on studies of regulation of capZ activity in other cell types (e.g., *20*) might be that the high concentrations of PIP_2 in erythrocytes (50 μM; *52*) could reduce the apparent affinity of cytosolic EcapZ for actin filament barbed ends, thus changing the apparently stronger capping protein into the weaker one. For example, we estimate that at the 50 μM PIP_2 concentration calculated for intact erythrocytes, the K_{cap} of EcapZ would be about 575 nM, well above the cytosolic EcapZ concentration of 100−200 nM that we determined for erythrocytes. PIP_2 has no effect on the capping activity of adducin (P. A. Kuhlman and V. M. Fowler, unpublished data). However, a problem with this explanation is that the ability of EcapZ to bind to actin filament barbed ends in 5P8 ghosts but not Mg^{2+} ghosts is not correlated with a reduction in the level of PIP_2 in the 5P8 ghosts. Other explanations are (1) a high off rate for capZ (*18*), which is not the case (*47*), or (2) molecular crowding in the cytoplasm, which might reduce the apparent affinity of capping protein for barbed filament ends (*21*). However, again, neither a high off rate nor molecular crowding effects can account for the inability of purified EcapZ to bind to the actin filament barbed ends in Mg^{2+} ghosts or in membranes prepared by saponin lysis in physiogical salt.

Adducin is also associated with the plasma membranes of a variety of nonerythroid cells (*10*, *60*), where it may also cap the barbed ends of short actin filaments in the membrane skeleton. In these cells, where capZ has been shown previously to be associated with cytoskeletal actin filaments, it may be that binding of adducin to spectrin−actin complexes serves to target adducin to the barbed ends of short actin filaments in the membrane skeleton while capZ binds to the barbed ends of a different population of (longer?) actin filaments extending into the cytoplasm. An additional unique feature of adducin as a barbed end capping protein is that adducin can also associate with the membrane protein, stomatin (*61*). This could provide an additional direct linkage of the short actin filaments to the plasma membrane, in addition to the previously described actin filament linkages via spectrin and protein 4.1 to the ankyrin/band 3 complex and to glycophorin C (*4*).

In platelets, thrombin stimulation leads to a transient rise in the amount of free actin filament barbed ends that is linked to a rise in PIP_2 levels (*62*) and a concurrent dissociation of platelet capZ, thus leading to stimulation of actin polymerization (*19*, *20*). By analogy, it is tempting to speculate that, in some cells, specific signaling events might lead to downregulation of adducin's capping activity by calcium−calmodulin (*9*), and/or by phosphorylation or dephosphorylation reactions (*59*). This might result in selective uncapping of actin filament barbed ends in the spectrin−actin membrane skeleton and stimulation of actin polymerization. Displacement of adducin from actin filament barbed ends by capZ could also lead to detachment of filament ends from adducin−stomatin membrane linkages and consequent rearrangements of the membrane skeleton. In erythrocytes, this might be expected to have functional consequences for cellular deformability and the mechanical stability of the cells.

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REFERENCES

1. Weeds, A,. and Maciver, S. (1993) *Curr. Opin. Cell Biol. 5*, 63.
2. Schafer, D. A., and Cooper, J. A. (1995) *Annu. Rev. Cell Dev. Biol. 11*, 497.
3. Shen, B. W., Josephs, R., and Steck, T. L. (1986) *J. Cell Biol. 102*, 997.

4. Lux, S. E., and Palek, J. (1995) in *Blood, Principles and Practice of Hematology* (Handin, R. I., Lux, S. E., and Stossel, T. P., Eds.) p 1701, J. B. Lippincott Co., Philadelphia, PA.
5. Fowler, V. M. (1996) *Curr. Opin. Cell Biol. 8*, 86.
6. Fowler, V. M. (1997) *Cytoskeletal Regulation of Membrane Function*, pp 79−89, The Rockefeller University Press, New York.
7. Weber, A., Pennise, C. R., Babcock, G. G., and Fowler, V. M. (1994) *J. Cell Biol. 127*, 1627.
8. Gregorio, C. C., Weber, A., Bondad, M., Pennise, C. R., and Fowler,V. M. (1995) *Nature 377*, 83.
9. Kuhlman, P. A., Hughes, C. A., Bennett, V., and Fowler, V. M. (1996) *J. Biol. Chem. 271*, 7986.
10. Gilligan, D. M., and Bennett, V. (1993) *Semin. Hematol. 30*, 74.
11. Byers, T. J., and Branton, D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6153.
12. Pinder, J. C., Weeds, A. G., and Gratzer, W. B. (1986) *J. Mol. Biol. 191*, 461.
13. Tsukita, S., Tsukita, S., and Ishikawa, H. (1984) *J. Cell Biol. 98*, 1102.
14. Podolski, J. L., and Steck, T. L. (1988) *J. Biol. Chem. 263*, 638
15. Gardner, K., and Bennett ,V. (1986) *J. Biol. Chem. 261*, 1339.
16. Gardner, K., and Bennett, V. (1987) *Nature 328*, 359.
17. Mische, S. M., Mooseker, M. S., and Morrow, J. S. (1987) *J. Cell Biol. 105*, 2837.
18. DiNubile, M. J., Cassimeris, L., Joyce, M., and Zigmond, S. H (1995) *Mol. Biol. Cell 6*, 1659.
19. Nachmias, V. T., Golla, R., Casella, J. F., and Casella, E. B. (1996) *FEBS Letts. 378*, 258.
20. Barkalow, K., Witke, W., Kwiatkowski, D. J., and Hartwig, J. H. (1996) *J. Cell Biol. 134*, 389.
21. Hug, C., Jay, P. Y., Reddy, I., McNally, J. G., Bridgman, P. C., Elson, E. L., and Cooper, J. A. (1995) *Cell 81*, 591.
22. Lin, D. C., and Lin, S. (1978) *J. Biol. Chem. 253*, 1415.
23. Schafer, D. A., Korshunova, Y. O., Schroer, T. A., and Cooper, J. A. (1994) *J. Cell Biol. 127*, 453.
24. Fowler, V. M (1990) *J. Cell Biol. 111*, 471.
25. Ursitti, J. A., and Fowler, V. M. (1994) *J. Cell Sci. 107*, 1633.
26. Bennett, V. (1983) *Methods Enzymol. 96*, 313.
27. Fowler, V. M., and Bennett, V. (1984) *J. Biol. Chem. 259*, 5978.
28. Laemmli, U. K. (1970) *Nature 227*, 680.
29. Fowler, V. M. (1987) *J. Biol. Chem. 262*, 12792.
30. Towbin, H., Staehelin, T., and Gorden, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.
31. Davis, J., and Bennett. V. (1982) *J. Biol. Chem. 257*, 5816.
32. Casella, J. F., and Cooper, J. A. (1991) *Methods Enzymol. 196*, 140.
33. Caldwell, J. E., Heiss, S. G., Mermall, V., and Cooper, J. A. (1989) *Biochemistry 28*, 8506.
34. O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
35. Granger, B. L., Repasky, E. A., and Lazarides, E. (1982) *J. Cell Biol. 92*, 299.
36. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol. 85*, 164.
37. Fowler, V. M., and Taylor, D. L. (1980) *J. Cell Biol. 85*, 361.
38. Fowler, V. M., and Pollard, H. B. (1982) *J. Cell. Biochem. 18*, 295.
39. Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33.
40. Weber, A., Northrop, J., Bishop, M. F., Ferrone, F. A., and Mooseker, M. S. (1987) *Biochemistry 26*, 2528.
41. Casella, J. F., Maack, D. J., and Lin, S. (1986) *J. Biol. Chem. 261*, 10915.
42. Lin, D. C., and Lin, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2345.
43. Kilimann, M. W., and Isenberg, G. (1982) *EMBO J. 1*, 889.
44. Steck, T. L., and Kant, J. A. (1974) *Methods Enzymol. 31*, 172.
45. Schafer, D. A., Waddle, J. A., and Cooper, J. A. (1993) *Cell Motil. Cytoskeleton 25*, 317.
46. Cooper, J. A., Blum, J. D., and Pollard, T. D. (1984) *J. Cell Biol. 99*, 217.
47. Schafer, D. A., Jennings, P. B., and Cooper, J. A. (1996) *J. Cell Biol. 135*, 169.
48. Maun, N. A., Speicher, D. W., DiNubile, M. J., and Southwick, F. S. (1996) *Biochemistry 35*, 3518.
49. Haus, U., Hartmann, H., Trommler, P., Noegel, A. A., and Schleicher, M. (1991) *Biochem. Biophys. Res. Commun. 181*, 833.
50. Heiss, S. G., and Cooper, J. A. (1991) *Biochemistry 30*, 8753.
51. Janmey, P. A. (1994) *Annu. Rev. Physiol. 56*, 169.
52. Ferrell, J. E., and Huestis, W. H. (1984) *J. Cell Biol. 98*, 1992.
53. Chilvers, E. R., Batty, I. H., Chaliss, R. A. J., Barnes, P. J., and Nahorski, S. T. (1991) *Biochem. J. 275*, 373.
54. Maruyama, K., and Ohashi, K.(1978) *J. Biochem. (Tokyo) 84*, 1017.
55. Wegner, A. (1982) *J. Mol. Biol. 161*, 217.
56. Cooper, J. A. (1987) *J. Cell Biol. 105*, 1473.
57. Hart, M. C., Korshunova, Y. O., and Cooper, J. A. (1997) *Cell Motil. Cytoskelton. 38*, 120.
58. Casella, J. F., Craig, S. W., Maack, D., and Brown, A. E. (1987) *J. Cell Biol. 105*, 371.
59. Matsuoka, Y., Hughes, C. A., and Bennett, V. (1996) *J. Biol. Chem. 271*, 25157.
60. Kaiser, H. W., O'Keefe, E., and Bennett, V. (1989) *J. Cell Biol. 109*, 557.
61. Sinard, J. H., Stewart, G. H., Argent, A. C., and Morrow, J. S. (1994) *Mol. Biol. Cell. 5*, 421a.
62. Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, T. P (1995) *Cell 82*, 643.
63. Fenner, C., Traut, R. R., Mason, D. T., and Wikman-Coffelt, J. (1975) *Anal. Biochem. 63*, 595.

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