Purification and Properties of a Ca^{2+}-Independent Barbed-End Actin Filament Capping Protein, CapZ, from Human Polymorphonuclear Leukocytes†

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ABSTRACT: In human polymorphonuclear leukocytes (PMN), changes in the actin architecture are critical for the shape changes required for chemotaxis and phagocytosis. Barbed-end capping proteins are likely to regulate actin assembly in PMN. The previously identified barbed-end blocking proteins in PMN, gelsolin and CapG, require Ca^{2+} to initiate capping of actin filaments. Because chemoattractants can stimulate PMN actin assembly by a calcium-independent signal transduction pathway, we sought to purify a calcium-independent barbed-end capping activity from PMN cytoplasmic extracts. A Ca^{2+}-insensitive actin polymerization inhibitory activity was partially purified from human PMN [Southwick & Stossel (1981) *J. Biol. Chem 256*, 3030]. Using five column chromatography steps, we purified the protein to homogeneity as assessed by silver staining. Purification was associated with an increase in specific activity of greater than 40 X. Western blot analysis identified the protein as the nonmuscle isoform of the heterodimeric capping protein capZ. Human PMN capZ has an apparent disassociation constant of 3 nM for capping in the presence or absence of micromolar Ca^{2+}, as assessed by both pyrenylactin elongation and depolymerization assays. Similar to the the activity reported for the actin polymerization inhibitor, activity of PMN capZ was inhibited by increasing the KCl concentration from 0.1 M to 0.6 M. The capping function was also inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2) micelles, with half-maximal inhibition occurring at 5.5 μg mL^{-1}. PMN capZ did not nucleate actin assembly, sequester actin monomers, or sever actin filaments. Quantitative Western blot analysis revealed that capZ levels corresponded to 0.7−1.0% of the total human PMN cytoplasmic protein. Given its abundance and high affinity for barbed filament ends, capZ is likely to play an important role in the calcium-independent regulation of actin filament assembly associated with PMN chemotaxis.

Polymorphonuclear leukocyte (PMN)[1] chemotaxis, phagocytosis, and degranulation require a coordinated rearrangement of the cell's peripheral cytoskeleton (Southwick & Stossel, 1983). Actin is the predominant cytoskeletal protein found in PMN, representing approximately 15% of total cytoplasmic protein. During cell movement, actin monomers rapidly associate onto the "barbed" or high-affinity ends of actin filaments. Two families of actin "barbed-end" capping proteins have been previously described: the gelsolin/villin/capG (MCP, gCap39), and the capZ/capping protein family. The gelsolin/villin/capG family requires micromolar ionized calcium to function, while capZ function is calcium-independent (Hartwig & Kwiatkowski, 1991; Casella *et al.*, 1986). The capping activity of proteins from both groups can be inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2) (Janmey & Stossel, 1987; Yu *et al.*, 1990; Cooper & Heiss, 1991). PIP_2 turnover rate is markedly increased upon PMN stimulation (Cockcroft *et al.*, 1985), and PIP_2 inhibition of capping activity may play a critical role in PMN actin-based motility (Stossel, 1993).

In 1981, an actin polymerization inhibitor was purified by ion exchange and gel filtration chromatography from cytoplasmic extracts of human PMN (Southwick & Stossel, 1981). This PMN actin polymerization inhibitor lowered the viscosity of actin in both high and low ionized calcium. Using five chromatography steps, we now show that this viscosity lowering activity is due solely to the 65 000 dalton heterodimeric protein capZ. This is the first report of capZ purified from mammalian phagocytic cells. Our functional studies demonstrate that PMN capZ strictly binds the barbed-ends of actin filaments, but has no severing, nucleating, or monomer sequestering activity. The capping activity of nonmuscle capZ is modulated by PIP_2. Quantitative Western blot analysis indicates that capZ represents 0.7−1.0% of the total protein in human PMN cytoplasmic extracts and can account for the majority of the calcium-insensitive barbed-end capping activity in PMN. The abundance of this phosphoinositide-regulated, Ca^{2+}-independent, high-affinity barbed-end capping protein suggests that capZ could play a prominent role in the regulation of actin assembly during PMN motile events.

MATERIALS AND METHODS

Isolation of Human Polymorphonuclear Leukocytes (*PMN*). Fresh leukocyte-enriched fractions from the whole blood of

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[1] Abbreviations: PMN, polymorphonuclear leukocytes; PIP_2, phosphatidylinositol 4,5-bisphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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healthy donors were obtained from the community blood bank and purified as previously described (Southwick & Stossel, 1981). For each preparation, 15−45 buffy coat units were used.

Purification of CapZ from Human PMN. The initial procedures which included ion exchange and gel filtration chromatograpy were identical to those described previously (Southwick & Stossel, 1981). Active fractions derived from gel filtration chromatography were pooled and dialyzed against a buffer containing 8 mM MES, pH 6.0, 1 mM sodium azide, and 0.1 mM PMSF (buffer S) and applied to a 5 mL Econo-Pac High S cation exchange column (Bio-Rad Laboratories, Inc., Hercules, CA) equilibrated with buffer S. The column was washed with 5 mL of buffer S, followed by a 30 mL (1 mL/min) linear salt gradient to 0.150 M KCl in buffer S. Actin filament shortening activity was measured using falling ball microviscometry (MacLean-Fletcher & Pollard, 1980) for DEAE ion exchange and gel filtration fractions (Southwick & Stossel, 1981). However, to reduce the quantity of protein required to measure actin filament shortening activity, the band 4.1 capping assay was used for all subsequent purification steps (see Actin Polymerization and Depolymerization Studies below). Active fractions were pooled, dialyzed against S2 buffer (10 mM imidazole, pH 7.8, 1 mM DTT, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM EGTA), and applied to a Mono Q HR 5/5 anion exchange column (Pharmacia Biotech Inc., Piscataway, NJ) equilibrated with S2 buffer. The column was then washed with 5 mL (1 mL/min) of 0.1 M KCl in S2 buffer, and followed by a 12 mL linear gradient, 0.1−0.3 M KCl, in S2 buffer. As the final purification step, we utilized hydroxylapatite chromatography as previously described by Casella *et al.* (1986). Active fractions were dialyzed against a 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM DTT, applied to a 1 mL hydroxylapatite (Econo-Pac HTP, Bio-Rad Laboratories, Inc.) column equilibrated with the same buffer, and eluted with a linear gradient to 75 mM potassium phosphate, pH 7.0. Protein concentrations were measured using the Quantigold assay (Diversified Biotech, Boston, MA) and then stored at −20 °C in 30% ethylene glycol (under these storage conditions, the specific activity of the protein remained stable for >3 months). To assess the specific activity at each purification step, the spectrin−actin−4.1 nuclei assay was used (see below). The amount of total protein required to inhibit the elongation rate to half of the control rate was determined and this value divided into 1. For example, 0.025 mg of starting extract was required to inhibit the elongation rate of these nuclei by 50%. The specific activity of the starting extract therefore was $1/0.025\ \text{mg} = 40\ \text{mg}^{-1}$.

Amino Acid Sequencing. Peptides were electroblotted from SDS−polyacrylamide gels onto poly(vinylidene difluoride) (PVDF) membranes as previously described (Mozdzanowski *et al.*, 1992). Coomassie blue stained bands were excised, subjected to proteolytic digestion, and sequenced on an Applied Biosystems Model 475A sequencer using gas phase TFA delivery and an on-line Model 120A PTH analyzer with modifications as previously described (Reim & Speicher, 1992).

Actin Polymerization and Depolymerization Studies. Pyrene-actin was used for all kinetic studies, and all assays were performed at 25 °C. Fluorescence intensity was monitored using a Perkin-Elmer LS-5 fluorescence spectrophotometer with excitation and emission wavelengths of 364 and 407 nm, respectively. Actin filament depolymerization assays were performed in buffer containing 10 mM imidazole, pH 7.5, 0.5 mM ATP, 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM DTT (buffer P) also containing either a final concentration of 1 mM EGTA or 1 mM $CaCl_2$ as previously described (Southwick & DiNubile, 1986). Actin filament severing was performed using gelsolin-capped actin filaments as previously described (Southwick, 1995). The actin−spectrin nuclei elongation assays using red blood cell derived spectrin/band 4.1/actin nuclei were performed as previously described (Casella *et al.*, 1986; Young *et al.*, 1994). These complexes contain only free barbed actin filament ends (Lin & Lin, 1978). The capZ and spectrin/band 4.1/actin nuclei were allowed to incubate for 2 min prior to the addition of the pyrene-actin. Steady-state G- and F-actin concentrations were determined as previously described (Northrop *et al.*, 1986). To examine nucleation activity, 1.5 μM pyrene-labeled G-actin (gel-filtered immediately prior to use) was allowed to polymerize in buffer P in the presence of varying concentrations of capZ and the assembly rate monitored by fluorimetry (Southwick & DiNubile, 1986). Monomer sequestering activity was measured using unlabeled gelsolin-capped actin filaments (gelsolin:actin molar ratio, 1:16) as previously described (Young *et al.*, 1990). Pyrene-labeled G-actin (final concentration 0.8 μM; 50% and 100% labeled) was added to buffer P containing the gelsolin/actin nuclei (final concentration 15 nM/0.24 μM) and varying concentrations of capZ.

Lipid Inhibition Studies. Phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) and used without further purification. Phosphatidylinositol 4,5-bisphosphate (PIP_2) was obtained from Calbiochem (Calbiochem, LaJolla, CA). The phospholipids PIP_2, PI, PC, and PS were treated as previously described (Janmey & Stossel, 1989). The inhibition of capZ capping was assessed by measuring the initial slopes of depolymerization in the presence of varying amounts of each lipid.

Other Methods. Actin was purified from rabbit skeletal muscle by the method of Spudich and Watt (1971) and gel-filtered through a Superdex 200 16/60 (Pharmacia Biotech Inc., Piscataway, NJ) column for polymerization kinetic studies. Pyrenylactin was prepared according to the method of Kouyama and Mihashi (1981) with the modifications previously described (Northrop *et al.*, 1986). Protein samples were subjected to electrophoresis using 10% SDS−PAGE. Proteins were visualized using Coomassie brilliant blue R-250 or Silver (Silver stain plus kit, Bio-Rad Laboratories, Inc.). Western blots were preformed using standard protocols (Towbin *et al.*, 1979). Blots were developed as described by Dabiri *et al.* (1992), or using Amersham's enhanced chemiluminescence (ECL) technique (Amersham Life Science, Inc., Arlington Heights, IL) . Primary antibodies used included anti-human annexin VI monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA), rabbit anti-human annexin VI antibody (kind gift from Joel Ernst, University of California, San Fransico), rabbit anti-human L-plastin antibody, and rabbit anti-chicken skeletal muscle capZ α and β (kind gift from John Cooper, Washington University, St. Louis, MO).

RESULTS

Identification of the PMN Actin Polymerization Inhibitor. The Ca^{2+}-insensitive actin filament shortening activity (previously named PMN actin polymerization inhibitor) was purified by ion exchange chromatography and gel filtration, and resulted in the enrichment of 62 and 65 kDa polypeptides. Yields of the activity and the purity of the two polypeptides were identical to the previously reported purifications (Southwick & Stossel, 1981). Densitometry scans of Coomassie blue stained gels suggested the two polypeptides represented >90% of the total protein. However, subsequently silver-stained SDS−PAGE of the active fractions revealed substantial quantitities of two additional polypeptides with molecular masses of 36 and 32 kDa that were not visualized by Coomassie blue staining (see below).

To determine the identity of the 62 and 65 kDa polypeptides, the active fractions were separated by SDS−PAGE and transferred to an Immobilon membrane. The 62 kDa polypeptide was subjected to trypsin digestion, and amino acid sequencing yielded sequences of 9 and 15 amino acids that were identical to amino acids 67−75 and 516−530, respectively, of human L-plastin (Lin *et al.*, 1988). Two fragments from BNPS-skatole digestion of the 65 kDa polypeptide yielded sequences of 18 and 23 amino acids that were identical to amino acids 193−210 and 344−366, respectively, of human annexin VI (Sudhof *et al.*, 1988; Crompton *et al.*, 1988). The identities of the 62 and 65 kDa polypeptides as L-plastin and annexin VI were further confirmed by western blot analysis (see Materials and Methods) (data not shown). The anti-human L-plastin antibody also cross-reacted with a 55 000 dalton polypeptide. A similar L-plastin degradation product has previously been observed in lymphocytes (Pacaud & Derancourt, 1993).

In order to determine whether L-plastin and annexin VI together, or individually, were responsible for the actin filament shortening activity, additional purification steps were undertaken. Because we had determined that the filament shortening activity measured by viscosity assays was the result of barbed-end capping activity (see below), all subsequent purification steps utilized spectrin−4.1 nuclei from human red blood cells and pyrenylactin to assay inhibitory activity (see Materials and Methods). Viscosity lowering activity was found to correlate exactly with the ability to block barbed-end actin filament assembly from spectrin−4.1−actin nuclei. Annexin VI and L-plastin were first separated by High S cation exchange chromatography, and this step removed the barbed-end blocking activity from the annexin VI fractions. Fractions with inhibitory activity were then applied to a Mono Q anion exchange column and eluted with a linear 0.1−0.3 M KCl gradient. Despite the presence of PMSF in dialysis solutions and the maintenance of all procedures at 4 °C, a 55 kDa L-plastin degradation product was formed in significant amounts at this stage of the purification. Mono Q chromatography separated the barbed-end capping activity from the 62 L-plastin fractions, but not from the 55 kDa degradation product. Significantly, in addition to the 55 kDa polypeptide, the active fractions also contained 36 kDa, and 32 kDa polypeptides which were clearly visualized upon silver staining of the SDS−PAGE. The final step, hydroxylapatite, successfully separated the L-plastin degradation product from these 36 and 32kDa polypeptides (Figure 1). All barbed-end capping activity copurified with the 36/32 kDa polypeptides. Using an antimuscle capZ polyclonal antibody, Western analysis identified the 36 and 32 kDa bands as capZ (Figure 2).

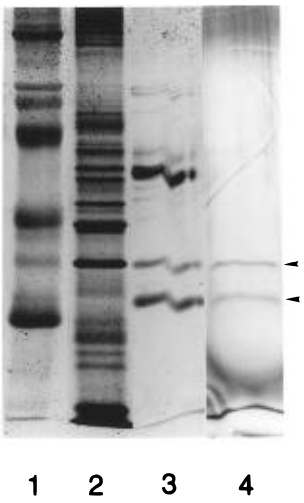

FIGURE 1: Silver-stained peak fractions from Mono Q and HA chromatography. High molecular weight standard (5 μg; lane 1), PMN extract (14 μg; lane 2), Mono Q column peak activity (2 μg; lane 3), and hydroxylapatite column peak activity (0.25 μg; lane 4) were subjected to 10% SDS−PAGE and silver-stained. Arrowheads point to the 36 kDa (α) and 32 kDa (β) subunits of capZ. Molecular weight standards used were 205×10^3, 116×10^3, 97.4×10^3, 66×10^3, 45×10^3, and 29×10^3, respectively.

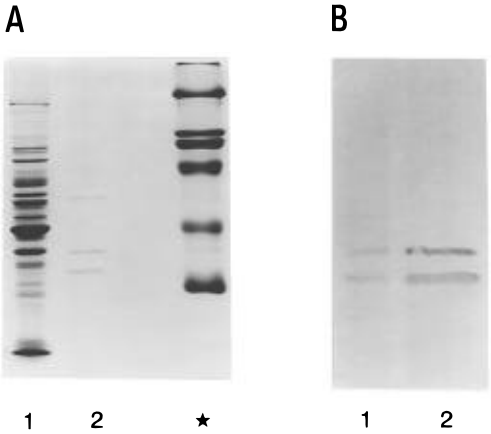

FIGURE 2: Western blot analysis of capZ purified by Mono Q chromatography. PMN extract (30 μg; lane 1) and the most active barbed-end capping fraction from Mono Q chromatography (3 μg; lane 2) were separated by 10% SDS−PAGE, and either stained (panel A) with Coomassie blue or subjected to Western analysis (panel B) probing with antisera to chicken skeletal muscle capZ and developed using a standard nitro blue tetrazolium and 5-bromo-4 chloro-3-idolyl phosphate method as previously described (Dabiri et al., 1992). The star identifies the lane containing the same high molecular weight standard used in Figure 1. Note the weak Coomassie blue staining of two capZ subunits in panel A, lane 2, as compared to the same fraction visualized by silver staining on lane 3 of Figure 1. The hydroxylappatite fraction, lane 4 of Figure 1, was not used for the Western blot analysis because the protein concentration was too low to allow detection by the nitro blue tetrazolium method (sensitivity approximately 0.5 μg). Attempts at concentrating this material led to excessive loss of protein.

Quantitation of CapZ in Human PMN and Estimation of Purification Yields. A linear standard curve was generated using known concentrations of highly purified PMN capZ, and these values were compared to the 36 and 32 kDa reactive bands in four different dilutions of PMN extracts (Figure 3). CapZ was determined to represent 0.7−1% of the total protein in human PMN extracts, and the molar ratio

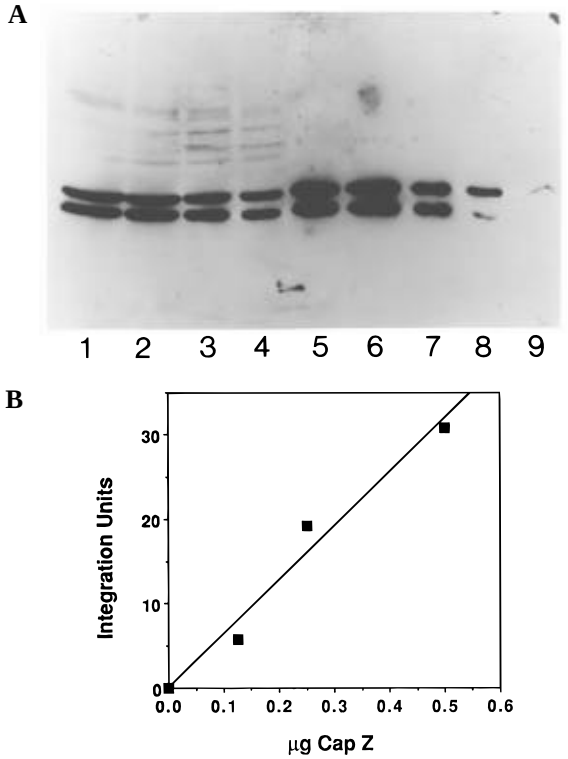

FIGURE 3: Quantitation of capZ in human PMN cytoplasmic extracts. (A) Autoradiogram of nitrocellulose transfers probed with anti-chicken skeletal muscle capZ, and developed by chemiluminescence to increase sensitivity (note the much stronger reactions in this figure as compared to Figure 2B). Lanes 1−4, decreasing concentrations of human PMN extracts: 75, 50, 25, and 12.5 μg, respectively. Lanes 5−9, decreasing concentrations of purified human capZ: 1.0, 0.5, 0.25, 0.13, and 0.065 μg, respectively. Human PMN were purified by ficoll-hypaque centrifugation. Note that as the amount of PMN extract was increased, the lane broadened, making exact quantitation of capZ in lanes 1 and 2 less accurate. However, comparisons of densitometry scans of lanes 3 and 4 with known amounts of purified protein (lanes 5−9) demonstrated that capZ represented 0.7−1% of the total protein. The antibody only weakly interacted with the lowest concentration of purified capZ (lane 9) detecting only the α 36kDa subunit. (B) Graphic plot of the densitometry scans of lanes 6−8 in panel A demonstrates a linear relationship between density (integration units) and micrograms of purified protein ($r = 0.97$). Concentrations above 0.5 μg saturated the reaction. Note the equal intensities of lanes 5 (1 μg) and 6 (0.5 μg) in panel A. The densities of lanes 1−4 were within the linear range of the standard curve.

of capZ to actin monomers was estimated to be 1 to 25. The quantity of PMN extract used for our purifications ranged from 350−700 mg. Therefore, starting quantities of capZ ranged from 2.5−7 mg. Yields following four column purification steps (DEAE ion exchange, gel filtration, Mono S, and Mono Q) ranged from 0.04−0.32 mg or approximately 1.5−4.5% of the initial material. The final hydroxylapatite column resulted in a significant loss of protein, yielding 4−5 μg of highly purified capZ. CapZ failed to elute from the hydroxylapatite column as a sharp peak, causing considerable dilution of the protein. The five purification steps achieved an overall enrichment of 40 times the initial specific activity, the specific activity increasing from 40 inhibitor units/mg to a final value of 1550 units/mg.

Barbed-End Capping Activity. As shown in Figure 4, capZ slowed the rate of actin filament disassembly in a concentration-dependent fashion. The marked slowing in the depolymerization rate was most consistent with blocking of monomer release from the barbed-ends of actin filaments.

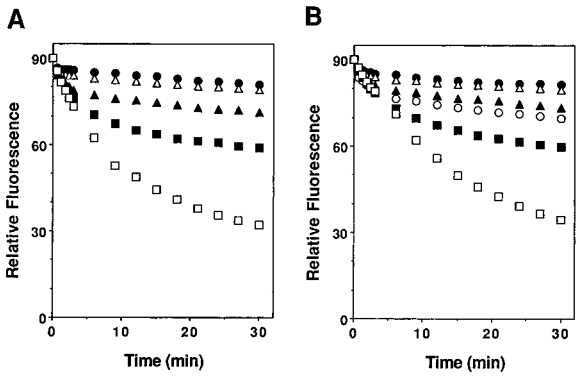

FIGURE 4: Effects of purified capZ on actin filament depolymerization. Pyrene-actin (2 μM) was allowed to polymerize to steady state in the presence of 1 mM EGTA (panel A) or 1 mM $CaCl_2$ (panel B). At time zero, aliquots of F-actin were diluted 1:40 (final concentration 50 nM) into varying concentrations of purified PMN capZ in buffer P containing EGTA (panel A) or 1 mM $CaCl_2$ (panel B) and fluorescence intensity was monitored over time. The final CapZ concentrations in panel A were 215 nM (closed circles), 27 nM (open triangles), 13.6 nM (closed triangles), 3.4 nM (closed squares), and 0 nM (open squares). In panel B, the CapZ concentrations were 108 nM (closed circles), 36 nM (open triangles), 18 nM (closed triangles), 9 nM (open circles), 3.6 nM (closed squares), and 0 nM (open squares).

The apparent dissociation constant for the interaction of capZ with the barbed-end ($K_{D,app}$) was approximately 3 nM. CapZ inhibited actin depolymerization to a similar extent in the presence and absence of Ca^{2+} (compare Figure 4A and Figure 4B). The ability of the PMN actin depolymerization inhibitor to decrease the viscosity of actin filament solutions was previously reported to be inhibited by increasing salt concentrations from 0.1 M KCl to 0.6 M KCl (Southwick & Stossel, 1981). Therefore, the effect of increasing salt concentration on the barbed-end capping activity of capZ was also assessed by the depolymerization assay. Capping activity was progressively inhibited as the KCl concentration in buffer P was raised to 0.6 M. This highest salt concentration caused near-complete inhibition of capZ capping activity (data not shown). Human PMN capZ's ability to block barbed-end monomer addition was examined using a complex isolated from red blood cells composed of actin, band 4.1, and spectrin (Lin & Lin, 1978; Casella *et al.*, 1986). As shown in Figure 5, capZ blocked barbed-end actin filament assembly in a concentration-dependent manner. The apparent dissociation constant ($K_{D,app}$), 3.0 nM, was identical to that determined from our depolymerization studies. Capping activity was not affected by changing the Ca^{2+} concentration (data not shown).

As expected with a barbed-end capping protein, substoichiometric concentrations of capZ increased the critical concentration of monomeric actin from 0.3 to ~0.8 μM (data not shown).

Nucleation Assay. CapZ nucleation activity was also tested in the presence and absence of calcium. Concentrations of capZ up to 219 nM (see Figure 6A, closed squares) failed to stimulate the polymerization of actin as compared to actin monomers alone (open squares). Addition of unlabeled actin filaments 10 min after addition of salt caused a rapid rise in pyrenylactin fluorescence indicating the actin was capable of assembling (closed triangles). Figure 6A is representative of multiple assays all of which demonstrated a failure to stimulate actin assembly. In two instances, capZ

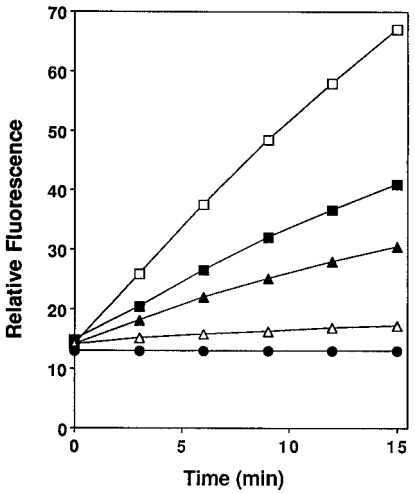

FIGURE 5: Effects of purified capZ on actin filament polymerization from spectrin/band 4.1/actin nuclei. To a constant (1.25 μg/mL) amount of spectrin/band 4.1/actin nuclei was added, 0.55 μM pyrene-labeled G-actin to polymerize in the presence of varying concentrations of capZ in buffer P containing 1 mM EGTA. The capZ and spectrin/band 4.1/actin nuclei were allowed to incubate for 2 min prior to the addition of pyreneactin. The concentration of pyreneactin used (0.55 μM) failed to spontaneously nucleate during the time period of the experiments. This concentration was also below the critical concentration of the pointed ends. Therefore, the rise in fluorescence reflected growth strictly from the barbed-ends of the actin filament complexes. The final concentrations of CapZ in the solutions were 18 nM (closed circles), 9 nM (open triangles), 3.6 nM (closed triangles), 1.8 nM (closed squares), and 0 nM (open squares).

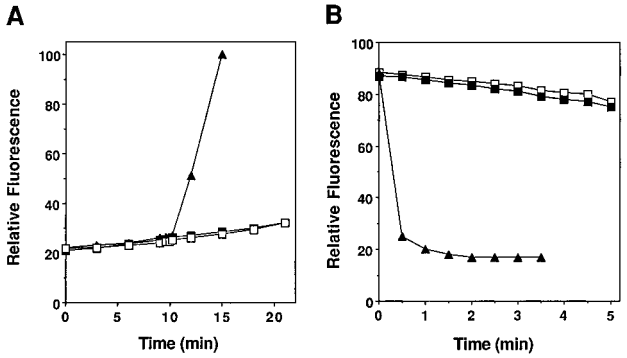

FIGURE 6: Effects of PMN capZ on G-actin nucleation and actin filament severing. (A) At time zero, a final concentration of 0.1 M KCl and 1 mM $MgCl_2$ was added to 1.5 μM gel-filtered, pyrene-labeled G-actin combined with a final concentration of 215 nM capZ (closed squares) or in buffer P alone (open squares). These two curves virtually overlie each other, and control points were taken corresponding to every experimental point. Identical effects were seen in the presence of 1 mM $CaCl_2$. The ability of the pyrene-labeled actin to polymerize was assessed by adding a final concentration of 1.4 μM unlabeled F-actin to the control reaction 10 min after initiation of actin assembly by the addition of salt (closed triangles). (B) Gelsolin and pyreneactin (2 μM) were copolymerized (molar ratio 1:200 gelsolin to G-actin) to steady state in buffer P and 1 mM $CaCl_2$, forming filaments capped at the barbed-ends. These filaments were then diluted to a final actin concentration of 100 nM in buffer alone (open squares), buffer containing a final concentration of 215 nM capZ (closed squares), and buffer containing a final concentration of 100 nM gelsolin (closed triangles).

prolonged the lag phase and was associated with assembly rates that were slower than actin alone (data not shown).

Severing Assay. To examine the ability of capZ to break apart preformed actin filaments, gelsolin-capped filaments were diluted into a buffer containing varying concentrations

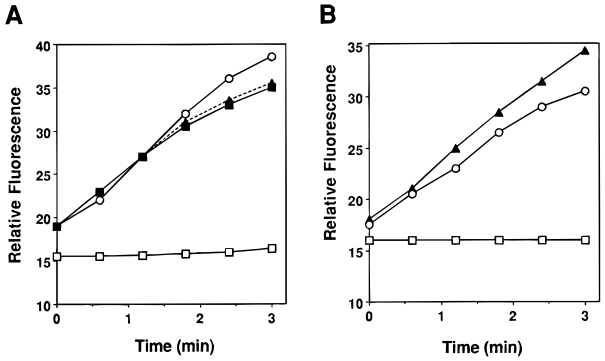

FIGURE 7: Effects of capZ on polymerization from gelsolin−actin nuclei. Pyrene-labeled G-actin (final concentration of 0.8 μM; 100% labeled) was polymerized in the presence of gelsolin−actin nuclei (molar ratio 1:16), and assembly rates were monitored in the presence of final concentrations of 290 nM capZ (closed squares) or 360 nM capZ (closed triangles) or in the presence of buffer alone (open circles). The same concentration of pyrene-G-actin alone failed to polymerize during the time period of our assay (open squares). The reaction was performed in the presence of either 1 mM EGTA (panel A) or 0.5 mM calcium (panel B). Similar results were obtained when this assay was repeated in the presence of G-actin that was 50% labeled.

of capZ. CapZ at concentrations as high as 215 nM did not accelerate the depolymerization rate (see Figure 6B, closed squares) as compared to filaments diluted into buffer (open squares). Dilution of gelsolin-capped actin filaments into a buffer containing free gelsolin (100 nM) caused a marked acceleration in the depolymerization rate (closed triangles).

Monomer Sequestration Assay. The ability of capZ to sequester monomeric actin was examined using gelsolin-actin nuclei. These nuclei possessing only free pointed ends were added to a mixture containing a fixed concentration of actin monomers and increasing concentrations of capZ. A monomer sequestering protein would be expected to slow the rate of actin assembly in a dose dependent manner (Young *et al.*, 1990). In the presence of 1 mM EGTA or 0.5 mM calcium, at final concentrations as high as 360 nM, capZ failed to alter the polymerization rate of gelsolin-actin nuclei (Figure 7). Identical effects were observed with varying mixtures of pyrene labeled and unlabeled monomeric actin.

Effect of PIP_2 on the Ability of CapZ To Bind the Barbed-Ends of Actin Filaments. The capping ability of 36 nM capZ was decreased in a concentration dependent fashion by the addition of PIP_2 micelles (Figure 8B). The half-maximal inhibition of capZ capping activity was observed at approximately 5.5 μg/mL (5 μM). At concentrations up to 11 μg/mL, the anionic phospholipids phosphatidylserine and phosphatidylinositol as well as the neutral phospholipid phosphatidylcholine failed to elicit a detectable decrease in capZ capping activity (Figure 8A).

DISCUSSION

Proteins that block the barbed-ends of actin filaments are likely to play a key role in maintaining the high actin monomer concentrations in unstimulated PMN, as well as regulating the rapid rise in F-actin associated with chemoattractant stimulation. In unstimulated PMN, the high concentration of unpolymerized actin (approximately 200 μM) is sequestered by two monomer binding proteins, thymosin β_4 and profilin. The relative high dissociation constants of

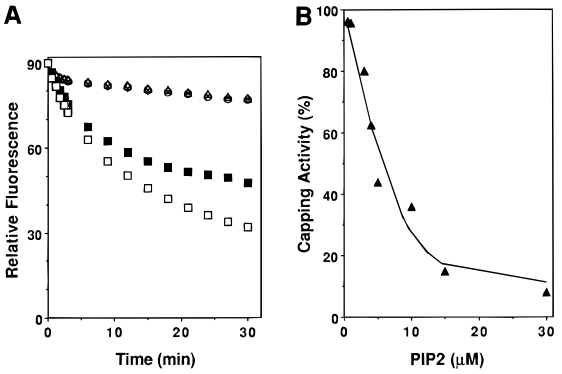

FIGURE 8: Effects of PIP_2 on capZ barbed-end capping activity. (A) Two micromolar (2 μM) pyrene-F-actin was diluted to a final concentration of 50 nM in buffer P containing 1 mM EGTA, 11 μg/mL phosphatidylinositol 4,5-bisphosphate (closed squares), phosphatidylcholine (open circles), phosphatidylserine ($\times$'s), or phosphatidylinositol (open triangles) combined with a final concentration of 36 nM capZ. The rates of depolymerization were compared to the same concentration of CapZ alone (+'s), and to uncapped actin filaments, i.e., F-actin depolymerized into buffer alone (open squares). (B) Barbed-end capping activity of 36 nM capZ was measured in the presence of varying amounts of phosphatidylinositol 4,5-bisphosphate (PIP_2). One hundred percent capping activity was defined as the inhibition of depolymerization observed with 36 nM capZ in the absence of PIP_2.

these sequestering proteins (K_Ds in the 2−5 μM range) indicate that free barbed-ends (K_D = 0.2 μM) can readily compete for sequestered actin monomers and induce the polymerization of a high percentage of PMN actin. Therefore, based on our present knowledge, the barbed-ends of actin filaments would need to be capped in unstimulated PMN in order to maintain the observed high concentrations of unpolymerized actin. However, in unstimulated PMN, intracellular Ca^{2+} concentrations are submicromolar (levels too low to activate the two barbed-end capping proteins previously purified from PMN, gelsolin and capG), strongly suggesting the existence of a barbed-end capping protein that remains active at low Ca^{2+} concentrations.

Within 30 s of exposure to a chemoattractant stimulus, PMN actin filament content doubles (Shalit *et al.*, 1987; Howard & Meyer, 1984; Fechheimer & Zigmond, 1983; Omann *et al.*, 1987; Lofgren *et al.*, 1993), F-actin content increasing from 40% to 80% of the total actin. Although chemoattractant stimulation is normally associated with a rise in intracellular Ca^{2+}, changes in intracellular Ca^{2+} are not required to induce chemoattractant-associated PMN actin assembly (Sha'afi *et al.*, 1986; Downey *et al.*, 1990; Sham *et al.*, 1993), again suggesting the existence of a barbed-end capping protein whose activity is not regulated by Ca^{2+}. Prior to our current findings, such a barbed-end capping protein had yet to be purified from mammalian phagocytic cells.

In 1981, one of us (F.S.S.) partially purified such an actin filament shortening activity called the PMN actin polymerization inhibitor. This activity was not affected by changing the calcium concentration, but could be completely inhibited by increasing the final KCl concentration to 0.6 M. Pyrenylactin assays were not available at that time, making further characterization of the activity difficult. We have now used pyrenylactin to characterize this activity and determined that the actin polymerization inhibitor is a barbed-end capping protein. The actin polymerization inhibitory activity was mistakenly attributed to the two most prominent polypeptides which migrated at 65 and 62 kDa on SDS−PAGE. This emphasizes the pitfalls in strategies to identify a relatively high-affinity actin regulatory protein, especially when actin filament assembly can be inhibited at substoichometric concentrations. The activity eluted on gel filtration with a Stokes radius of 32 Å and analytical centrifugation revealed an *s* value, 4.8 *S*, consistent with a 65 kDa monomeric protein. These findings initially suggested that a mixture of the 65 and 62 kDa monomeric proteins was responsible for the actin filament shortening activity found in PMN extracts.

Several observations subsequently led to the discovery of the true identity of the capping protein. First, amino acid sequence analysis demonstrated the 62 kDa peptide was not a degradation product of the 65 kDa protein, the lower molecular mass protein being identified definitively as L-plastin and the higher molecular mass protein as annexin VI. Neither of these proteins was known to cap the barbed-ends of actin filament. Our subsequent purification steps separated both of these proteins from the barbed-end capping activity, the activity copurifying with the 36/32 heterodimeric protein capZ. This protein possesses an isoelectric point and Stokes radius that are nearly identical to both L-plastin and annexin VI, explaining their copurification. Throughout our purification of the activity, human PMN capZ was poorly visualized by Coomassie blue staining, with its detection being greatly enhanced by silver staining or loading larger quantities of protein. Taken together, these properties explain our earlier inability to detect this heterodimeric barbed-end capping protein.

Functional characterization of human PMN capZ revealed an apparent dissociation constant for the barbed-end of actin filaments of 3 nM. This affinity is similar to capZ purified from brain and muscle (0.5 −10 nM) (Casella *et al.*, 1986; Caldwell *et al.*, 1989; Kilimann & Isenberg, 1982). Inhibition of monomer exchange at the barbed filament ends was not affected by changing the calcium concentration. However, as noted in the original description of the PMN actin polymerization inhibitor, increasing the KCl concentration to a final concentration of 0.6 M completely blocked PMN capZ function. In addition, capping could be inhibited by PIP_2 at half-maximal concentrations (5 μM), similar to those required to inhibit the skeletal muscle (Heis & Cooper, 1991) and *Dictyostelium* capping proteins (Haus *et al.*, 1991). We found that PMN capZ strictly functioned to cap actin filaments. The protein failed to sever actin filaments. Unlike gelsolin and capG which also bind to and sequester actin monomers, PMN capZ demonstrated no monomer-sequestering activity. In addition, PMN capZ failed to nucleate actin assembly. The inability to nucleate actin assembly was also oberved with cap 32/34 (also called agonactin) (Sauterer *et al.*, 1991; Eddy *et al.*, 1993; Haus *et al.*, 1993) purified from *Dictyostelium* but differs from brain and skeletal muscle capZ which shorten the lag phase of actin assembly consistently at concentrations of 50 nM (Kilimann & Isenberg, 1982; Caldwell *et al.*, 1989). Concentrations of PMN capZ as high as 200 nM failed to nucleate actin assembly.

Quantitative Western blots reveal that cytoplasmic extracts of human PMN contain high concentrations of capZ, amounting to 0.7−1.0% of the total cytoplasmic protein. Actin in these same extracts represents approximately 15% of the total protein. Therefore, the molar ratio of capZ to actin in PMN would be expected to be approximately 1:25. Given the high affinity of capZ for the barbed-ends of actin

filaments, there should be sufficient quantities of capZ to block all of the actin filaments in unstimulated PMN, allowing the monomer-sequestering proteins to maintain a high concentration of unpolymerized actin. Uncapping of the high-affinity barbed-ends in response to chemoattractant stimulation, a condition known to increase phosphoinositide turnover, may lead to the dissociation of capZ from actin filaments and explain the rapid conversion of monomeric actin to filamentous actin. Human PMN regulate the concentration of actin filaments in their peripheral cytoplasm as they rapidly migrate to sites of infection. The purification and characterization of human PMN capZ and the demonstration of high concentrations in the cytoplasm of PMN emphasize the likely importance of capZ in the dynamic regulation of PMN actin filament assembly.

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