Characterization of the actin filament capping state in human erythrocyte ghost and cytoskeletal preparations

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The narrow Gaussian-length-distribution of actin filaments forming the cytoskeleton of the human erythrocyte indicates the existence of strict mechanisms for length determination and maintenance. A similar regulation is achieved in striated muscle by the capping of both the ends of the thin filaments, which consequently prevents monomer exchange. However, the ability of erythroid cytoskeletal preparations to nucleate actin polymerization has led to the proliferation of the idea that at least the barbed ends of the actin filaments are uncapped. The mechanism by which the length of the filaments is thus maintained has been left open to debate. In an effort to resolve any doubt regarding length-maintenance in human erythrocytes we have characterized the capping state of the actin filaments in a number of different ghost and cytoskeletal preparations. Under conditions of

sufficiently high bivalent-cation concentration the actin filaments retain functional caps at both the barbed and pointed ends. Hence filament capping at both ends prevents redistribution of the actin monomer in a similar manner to that proposed for the thin filaments of striated muscle. Actin filament uncapping is apparently caused by the centrifugal shearing stress imposed during ghost preparation. The uncapping is more pronounced when the bivalent-cation concentration is reduced or when the membrane is removed by detergents. The effects of bivalent cations seem to be mediated through the erythroid protein spectrin, consistent with the hypothesis of Wallis et al. [Wallis, Babitch and Wenegieme (1993) *Biochemistry* **32**, 5045–5050] that the ability of spectrin to resist shearing stress is dependent on the degree of bound bivalent cations.

INTRODUCTION

The human erythrocyte actin cytoskeleton consists of short actin filaments of well-defined length [1], cross-linked into a quasi-hexagonal lattice by long flexible spectrin molecules [1,2]. The ease of preparation of erythrocyte ghosts and the high number of filament ends, compared with total actin, provides a relatively simple system with which to investigate actin-filament length regulation and maintenance. The long-term maintenance of a narrow gaussian filament-length distribution strongly suggests the curtailment of the dynamic nature of actin by the capping of both the barbed and the pointed ends (for a fuller discussion see Fowler [3]).

The actin filaments in human erythrocytes are known to be capped at the pointed ends by tropomodulin [4,5], a protein also found at the pointed ends of the thin filaments in striated muscle, where it has been shown to be the functional capping protein and to regulate actin filament length [6]. The situation at the barbed ends of the actin filaments is less clear as two barbed-end capping proteins have been identified. The first was a non-muscle homologue of CapZ [7] that exhibited a 1 nM capping affinity *in vitro* and was down-regulated by phosphatidylinositol 4,5-bisphosphate (PIP₂) in a similar manner to CapZ. However, under various lysis conditions the erythrocyte non-muscle isoform of CapZ (eCapZ) was never localized to the actin cytoskeleton. The cytosolic eCapZ exhibited capping activity, implying the presence of another barbed-end capping protein [7]. Subsequent investigation demonstrated barbed-end capping activity in the previously studied cytoskeletal protein adducin [8]. Adducin, first identified as a calmodulin binding protein [9] and later shown to be involved in the recruitment of spectrin to the short actin filaments

[10,11], is stoichiometrically localized to erythrocyte actin filaments. The association of adducin with the erythrocyte actin cytoskeleton, and the concurrent loss of adducin with the availability of binding sites for eCapZ, suggested an involvement of adducin in actin filament barbed-end capping [7].

Recently DiNubile has speculated on the existence of a third barbed-end capping protein [12], due to the apparent premature cessation in pyrene-actin polymerization in the presence of erythrocyte ghosts. The increasing number of capping proteins in the erythrocyte raises the question of redundancy. Are these proteins superfluous to the cell's requirements or does the increasing number indicate a more dynamic actin filament network than currently thought?

The present study investigates the actin filament capping state of erythrocyte ghosts and actin cytoskeletons prepared under various buffer conditions. The maintenance of a sufficiently high bivalent-cation concentration during hypotonic lysis and ghost preparation leads to little uncapping of either end of the actin filaments. Actin filament uncapping can be induced by either a reduction in the bivalent-cation concentration or by extraction of the membrane with detergent. The mechanistic target of the bivalent cation is still unknown, although preliminary data suggest a role for spectrin in the uncapping event.

EXPERIMENTAL

Antibodies

Rabbit anti-human spectrin [S1515] and anti-actin [A2066] polyclonal antibodies were purchased from Sigma–Aldrich (Poole, Dorset, U.K.).

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; eCapZ, non-muscle isoform of CapZ found in erythrocytes; 5T8 ghosts, erythrocyte membranes prepared by lysis in 5 mM Tris/HCl (pH 8.0) buffer containing protease inhibitors; Mg²⁺ ghosts, erythrocyte membranes prepared by lysis in 5 mM Tris/HCl (pH 8.0) buffer containing protease inhibitors and 3 mM Mg²⁺; TBS, Tris-buffered saline; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C.

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Proteins

Erythrocyte capping protein and adducin were purified essentially as described by Kuhlman and Fowler [7] and Hughes and Bennett [13], respectively. DNase I was obtained from Sigma (catalogue reference DN-EP), resuspended in storage buffer [50% (v/v) glycerol, 1 mM CaCl_2 , 0.5 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), 5 $\mu\text{g}/\text{ml}$ pepstatin A, 5 $\mu\text{g}/\text{ml}$ leupeptin and 2% (v/v) ethanol] and kept at -20°C as described by Podolski and Steck [14]. Actin was purified from a rabbit skeletal-muscle acetone powder, as described by Pardee and Spudich [15], with an additional purification step using a 100 cm $\times$ 1.6 cm Superose 6 (Pharmacia) gel-filtration column. Fractions were tested for the ability to produce high viscosity on polymerization (indicating lack of intrinsic nuclei) by using the falling-ball method [16,17]. Actin was pyrene-labelled with *N*-(1-pyrene)iodoacetamide (P-29; Molecular Probes Europe BV, Leiden, The Netherlands) using the method of Kouyama and Mihashi [18] with the modifications of Weber et al. [19]. Unincorporated label was removed by gel-filtration using a 30 cm $\times$ 0.5 cm Superdex 200 gel-filtration column and FPLC (Pharmacia). Actin/spectrin seeds, derived from the low ionic-strength extract of erythrocyte membranes, were prepared as described previously [20]. Protein concentrations were determined from the absorption coefficients: 26600 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 290 nm for actin; 22000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 344 nm for pyrene, using a correction factor of $[A_{290} - (0.127 \times A_{344})]/26600$ for the actin concentration; 85000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 280 nm [21] and 100500 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 279 nm for human erythrocyte capping protein and human erythrocyte adducin respectively.

Pyrene-actin polymerization assay

Actin polymerization was monitored by observing the increase in pyrene fluorescence on polymerization using an SLM 48000 fluorimeter. Pyrene fluorescence was excited at 365 nm using a slit width of 0.5 nm. Fluorescence was detected at 407 nm using a slit width of 8 nm. To limit photobleaching the highly focused excitation beam was defocused with a convex lens (focal length of 100 mm), prior to the sample cuvette (5 mm square). All actin polymerization experiments were maintained at 25°C by a circulating-water bath. Under low-salt conditions (2 mM KCl/0.8 mM MgCl_2) polymerization was primarily from the barbed filament end. Other experiments were performed at higher salt concentrations (0.1 M KCl/2 mM Mg^{2+}) to detect polymerization from both the barbed and pointed filament ends. The polymerization was initiated by the addition of pyrene labelled G-actin (5% labelled; final concentration 5 μM) that had been primed with Mg^{2+} as described previously [8]. The use of DNase I to inhibit actin filament polymerization at the pointed end [14] required a 10 min incubation at 25°C prior to initiation of actin polymerization.

Erythrocyte isolation and ghost preparation

Small amounts of human erythrocytes were prepared from 10 ml of freshly drawn blood by sedimentation, at unit gravity, through 4 vol. of 0.75% (w/v) dextran T-500 (Pharmacia) at room temperature in Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 7.5)/150 mM NaCl]. The cells were then washed three times in 10 vol. of TBS supplemented with 10 mM glucose, before being resuspended to a 20% (v/v) suspension, with respect to the packed-cell volume. The cells were stored on ice until required, and the erythrocyte concentration was determined by counting cells with the aid of a haemocytometer.

Erythrocyte ghosts were prepared by hypotonic lysis with an excess (30-fold) of either 5T8 buffer [5 mM Tris/HCl (pH 8.0)/5 mM β -mercaptoethanol/1 mM NaN_3 /0.5 mM AEBSF; 5T8 ghosts] or the same buffer supplemented with various concentrations of MgCl_2 (Mg^{2+} ghosts). Following 10 min on ice, to facilitate lysis, the ghosts were collected by centrifugation at 11951 *g* in a Sorvall SS34 rotor at 4°C for 10 min. The ghosts were washed a further three times with the same buffer, before being resuspended to a defined volume, hence defining the number of erythrocyte equivalents per ml. Ghosts prepared in the absence of Mg^{2+} were white, whereas the Mg^{2+} ghosts retained a pink hue, indicative of a small amount of retained haemoglobin.

Removal of Mg^{2+} from Mg^{2+} ghosts

Freshly prepared Mg^{2+} ghosts were either washed by repeated resuspension in 5T8 buffer and centrifugation at 11951 *g* at 4°C for 10 min in a Sorvall SS34 rotor, or by dialysis against an excess of 5T8 buffer. Samples of ghosts were extracted after each wash or after 1 h of dialysis and tested for actin nucleation activity in the pyrene-actin polymerization assay. Samples taken during the dialysis experiment were also treated with Triton X-100 (0.1% final concentration) and centrifuged at 48700 *g* for 10 min at 4°C in a TLA 100 rotor to remove the actin cytoskeletons. A fraction of the supernatant was solubilized in SDS sample buffer and separated by SDS/PAGE [7.5–15% (w/v) polyacrylamide] [22]. Following blotting on to nitrocellulose, the spectrin region was probed with an antibody to erythrocyte spectrin and detected using ^{35}S -labelled donkey anti-rabbit IgG serum (Amersham Pharmacia Biotech, Uppsala, Sweden). Each lane was quantified by excision of the region of nitrocellulose, solubilization in 5 ml of scintillation fluid (Opti-phase 'high safe' 2; Fisher Scientific Ltd., Loughborough, U.K.) and scintillation counted.

Preparation of detergent-solubilized actin cytoskeletons

Human erythrocytes were mixed with an equal volume of 0.6% detergent (either Triton X-100 or saponin) in TBS supplemented with 0.5 mM AEBSF, 5 $\mu\text{g}/\text{ml}$ pepstatin A and 5 $\mu\text{g}/\text{ml}$ leupeptin. The cells were gently pipetted to aid lysis and then centrifuged, at 11600 *g* for 5 min in a microfuge, through 2 vol. of a 20% sucrose solution [TBS, 20% (w/v) sucrose, 3 mM MgCl_2 , 0.5 mM AEBSF, 5 $\mu\text{g}/\text{ml}$ pepstatin A and 5 $\mu\text{g}/\text{ml}$ leupeptin]. All but 100 μl of the supernatant was carefully removed to prevent loss of cytoskeletons and then the pellet was resuspended to a final volume of 200 μl by the addition of 100 μl of similar buffer without sucrose (TBS, 3 mM MgCl_2 , 0.5 mM AEBSF, 5 $\mu\text{g}/\text{ml}$ pepstatin A and 5 $\mu\text{g}/\text{ml}$ leupeptin). Hence the final sucrose concentration was 10% (w/v).

RESULTS

The actin nucleating activity was measured in a number of ghost and cytoskeletal preparations. Under low-salt conditions (2 mM KCl/0.8 mM MgCl_2) nucleating activity could be demonstrated with actin/spectrin seeds, 5T8 ghosts, saponin and Triton X-100 skeletons. In each case the nucleating activity could be inhibited with a saturating amount of eCapZ (30 nM), a reagent specific for the actin filament barbed-end (Figure 1A). Mg^{2+} ghosts were effectively devoid of nucleating activity. Under higher salt conditions (0.1 M KCl/2 mM MgCl_2) the Mg^{2+} ghosts were weak nucleators (Figure 1B), but this activity was abolished by eCapZ (15 nM), indicating the presence of a very small number of uncapped barbed ends. The nucleating activity of actin/spectrin seeds and 5T8 ghosts was identical, in so far that a

Figure 1 Actin filament capping state characterization in ghost and cytoskeletal preparations

(A) Raw data (non-standardized) depicting the polymerization of pyrene-labelled actin under low-salt conditions (2 mM KCl/0.8 mM $MgCl_2$) nucleated by a variety of erythrocyte preparations. (B) Raw data (non-standardized) of identical experiments polymerized under high-salt conditions (0.1 M KCl/2 mM $MgCl_2$). The nucleating activity of the erythrocyte preparations was modulated with erythrocyte capping protein (CP), DNase I (D), both (CP/D) or neither (—). Concentrations of capping proteins for the different experiments were as follows: CP = 30 nM for the low-salt experiment and 15 nM for the high-salt experiment (reduction in the concentration under high-salt conditions was essential to prevent expression of its nucleating activity); D = 800 nM, except in the case of 5T8 ghosts under high-salt conditions when 2.5 μ M was used in order to rule out sequestration as the mechanism of action. RBCs, red blood cells.

proportion of the nucleating activity could be abolished by eCapZ. The remainder of the activity due to polymerization from the pointed end of the filaments could be abolished by treatment with DNase I. Saponin skeletons were similar to actin/spectrin seeds and 5T8 ghosts, except that the proportion of nucleating activity due to pointed ends was smaller. The ability of eCapZ to inhibit the nucleating activity of Triton X-100 skeletons completely indicates that only the barbed ends are uncapped. To ensure complete pointed-end capping, and to demonstrate that the effects of DNase I were not through the sequestration of actin monomers, 2.5 μ M DNase I was used in the high-salt experiments of the 5T8 ghosts (Figure 1B, second panel down). Note that 2.5 μ M DNase I alone was unable to inhibit actin polymerization which reached an equilibrium fluorescence that was half that of the 5T8 ghosts alone, consistent with the sequestration of approx. half of the 5 μ M G-actin present in the assay.

Figure 2 Effect of magnesium concentration on ghost actin filament capping state

(A) Equivalent samples of human erythrocyte ghosts prepared under a range of Mg^{2+} ion concentrations, separated by SDS/PAGE and stained with Coomassie Brilliant Blue R250. The positions of molecular mass standards and three proteins whose association with the erythrocyte ghosts was dependent on Mg^{2+} ion concentration are indicated. (B) The corresponding ghosts were also tested for their ability to nucleate actin polymerization in the pyrene-actin elongation assay (raw data). (C) By assuming the ghosts in the absence of Mg^{2+} were 100% uncapped, then the extent of capping in the other samples could be estimated from the initial polymerization rates.

Figure 3 Comparison of actin filament barbed-end capping between 5T8 and Mg^{2+} ghosts

(A) Pyrene-actin polymerisation assay demonstrating the ability of 40 nM erythrocyte capping protein to block the polymerization from the barbed ends of 5T8 ghosts. This capping activity can be inhibited by 20 μ M PIP_2 . Actin alone has no intrinsic polymerization under these conditions. (B) The same assay demonstrating the ability of 500 nM adducin to prevent polymerization from the barbed ends of the actin filaments in 5T8 ghosts. In this case the inhibition can be relieved using 50 μ M calmodulin. (C) A similar concentration of calmodulin has little effect on the nucleating activity of Mg^{2+} ghosts.

Preparation of human erythrocyte ghosts in a weak Tris buffer supplemented with increasing concentrations of $MgCl_2$ resulted in ghosts with increased actin filament capping (Figure 2). Samples of the resulting ghosts separated by SDS/PAGE (7.5–15% polyacrylamide) and stained with Coomassie Brilliant Blue indicated at least three proteins whose association with the

Figure 4 Effect of 5T8 and Mg^{2+} ghosts on the pyrene-actin polymerization assay

(A) 5T8 ghosts (0.65×10^8 or 1.3×10^8 cell equivalents) were added to the actin/spectrin seeded assay. Addition of polymerization salts initiated the actin polymerization. (B) Mg^{2+} ghosts (1.3×10^7 , 2.6×10^7 and 3.9×10^7 cell equivalents) also added to actin/spectrin nucleated actin polymerization assay. (C) Addition of the Mg^{2+} ghosts (3.9×10^7 cell equivalents) at the end (800 s) of the polymerization assay as compared with inclusion at the start (0 s).

ghost was apparently affected by the Mg^{2+} ion concentration (Figure 2A). Haemoglobin was one such protein, although its association with the actin cytoskeleton has no effect on the capping state of the actin filament barbed-end [7]. The increasing Mg^{2+} concentration also reduced the extent to which the ghosts were able to act as nuclei in a pyrene-actin polymerization assay (Figures 2B and 2C). A repeat experiment utilizing $CaCl_2$ in place of $MgCl_2$ yielded identical results (results not shown), indicating that the effect was not specific to Mg^{2+} ions.

The actin filament barbed ends in the 5T8 ghosts were

Figure 5 Conversion of Mg^{2+} ghosts into 5T8 ghosts

Samples, taken during a 6 h dialysis of Mg^{2+} ghosts with 5T8 buffer, extracted with 0.1% Triton X-100 and centrifuged to remove the actin cytoskeletons (see the Experimental section) were separated by SDS/PAGE and either stained with Coomassie Brilliant Blue or Western-blotted (WB) for spectrin and actin. The position of the alpha and beta subunits of spectrin, actin and several molecular mass markers are indicated. The percentage of spectrin and actin released has been determined against the total amounts in the sample, and the extent of barbed-end uncapping has been determined by assuming the 6 h dialysis point gave 100% uncapping.

effectively identical to those in actin/spectrin seeds with respect to the ability of both eCapZ and adducin to totally inhibit actin polymerization (Figures 3A and 3B). This inhibition could be down-regulated by PIP_2 and calmodulin respectively (Figures 3A and 3B). Treatment of Mg^{2+} ghosts with calmodulin under the same conditions did not yield any significant nucleating activity (Figure 3C).

In an effort to confirm the observations of DiNubile [12], both 5T8 (Figure 4A) and Mg^{2+} (Figure 4B) ghosts were added to an actin/spectrin nucleated pyrene-actin polymerization assay (Figure 4). In the case of the 5T8 ghosts, addition of 0.65×10^8 and 1.3×10^8 erythrocyte equivalents resulted in an increased rate of actin polymerization, consistent with the additional nuclei present (Figure 4A). The end-point of the polymerization was essentially the same, but for small effects caused by light-scattering by the ghosts, thus indicating that there was no untoward capping activity from 5T8 ghosts. The addition of Mg^{2+} ghosts did not lead to an increase in the rate of actin polymerization due to the actin filaments being capped (Figure 4B). The fluorescence at the end of the polymerization was reduced proportionally to the amount of ghosts added (Figure 4B). The fluorescence signal at the end of the polymerization assay was similar regardless of the time at which the Mg^{2+} ghosts were added (Figure 4C). The instantaneous drop in fluorescence signal on addition of the ghosts was consistent with an inner-filter effect caused by

the haemoglobin in the Mg^{2+} ghost sample. Measurement of the extent of actin polymerization at the end of each reaction indicated no dramatic increase in the amount of G-actin (results not shown), a result not consistent with the idea of premature polymerization termination due to capping.

The removal of Mg^{2+} ions from Mg^{2+} ghosts by extended dialysis (Figure 5) against 5T8 buffer resulted in the uncapping of the actin filament barbed ends. Samples were taken during the dialysis, treated with 0.1% Triton X-100 and centrifuged (48 700 g for 10 min at 4 °C) to remove the actin cytoskeletons. The amount of spectrin and actin in the supernatants was determined empirically, by Coomassie Brilliant Blue staining, or quantitatively, by Western blotting with antibodies specific for spectrin and actin. These proteins were apparent in the supernatants after 2–3 h of dialysis, increasing thereafter until the end of the assay at 6 h. At the end of the assay approx. half of the actin and spectrin was not associated in macromolecular complexes, indicating the slow disintegration of the cytoskeleton.

DISCUSSION

Using the nucleated pyrene-actin polymerization assay, the uncapping of both actin filament ends in erythrocyte ghosts and cytoskeletons prepared in the absence of bivalent cations (Figure 1) has been confirmed. This observation is consistent with several other studies [12,14,23–29]. However, the presence of bivalent cations during ghost preparation results in actin filaments capped at both ends (Figure 2), an observation consistent with the simplest mechanism for maintaining a narrow Gaussian-length-distribution, as is thought to be the case for the thin filaments of striated muscle.

The mode of actin filament uncapping is not clear. Generally, uncapping is dependent on the degree of physical stress the cells are subjected to; this effect is amplified by treatment with detergent or by reduction in bivalent-cation concentration. During ghost isolation excessive centrifugal force results in ghost fragmentation. This was particularly true of the 5T8 ghosts which seemed to be substantially more fragile than the Mg^{2+} or Ca^{2+} ghosts (results not shown). Similar effects were observed by Das and Mukherjee [31], although they ascribed the observation to the presence of a heterogeneous population of erythrocyte ghosts. Our initial 5T8 ghost preparations when centrifuged (at 38 724 g) exhibited a lower packed main pellet with a diffuse pellet on top. This effect was circumvented by reducing the centrifugal force, resulting in a homogeneous pellet with no residue in the supernatant. It is likely that the two membrane populations observed by Das and Mukherjee [31] were derived from centrifugal damage during erythrocyte ghost preparation, especially due to their use of lysis and wash buffers devoid of bivalent cations.

The 5T8 ghosts act identically to actin/spectrin seeds with respect to the ability of both eCapZ and adducin to cap the barbed ends of the actin filaments and be released by PIP_2 and calmodulin/ Ca^{2+} respectively (Figures 3A and 3B). In contrast, the actin filaments in Mg^{2+} ghosts are capped at the barbed ends, presumably by adducin [7], and at the pointed ends by tropomodulin [6]. However, treatment of Mg^{2+} ghosts with 50 μ M calmodulin resulted in only a marginal increase in the nucleating activity of the ghosts (Figure 3C). Under identical conditions, 50 μ M calmodulin was able to inhibit the capping activity of purified adducin on 5T8 ghosts (Figure 3B). There are two possible explanations for these observations. (1) The adducin in the Mg^{2+} ghosts is modified compared with purified adducin, possibly by phosphorylation. It is known that PKC phosphorylation of adducin on Ser⁷²⁶ prevents the binding of calmodulin

[32]. (2) Alternatively, the barbed-end capping is facilitated by an as yet undiscovered capping protein. A third barbed-end capping protein has been proposed by DiNubile [12], due to the apparent premature cessation of actin polymerization in the presence of ghosts. Addition of 5T8 (white) ghosts to actin/spectrin-seeded actin polymerization produces a higher initial polymerization rate, consistent with the increase in the number of nuclei (Figure 4A). The fluorescence end-point, however, is effectively identical to that in the absence of ghosts, apart from a small effect due to light-scattering by the ghosts. The addition of Mg^{2+} (pink) ghosts produces no real increase in the initial polymerization rate due to the ghosts being capped (Figure 4B). There is a significant reduction in the fluorescence at the end of the time course, which is dependent on the amount of ghosts added. We have ruled out the action of a capping protein for three reasons. (1) Haemoglobin in the ghosts absorbs the emitted fluorescent (407 nm) light strongly. (2) Quantification of the amount of unpolymerized actin (actin critical concentration) indicated no significant change in the presence of ghosts (results not shown). (3) Addition of the Mg^{2+} ghosts at the end of the time course led to the same final fluorescence; this instantaneous effect is unlikely to be consistent with capping protein kinetics, but simply an inner-filter effect [33]. This being the case, adducin is still the best candidate for the active barbed-end capping protein in erythrocytes.

Treatment of Mg^{2+} ghosts with bivalent cation-free buffer (5T8 buffer), either by repeated washing (results not shown) or by extended dialysis (Figure 5), renders them good nuclei in the actin polymerization assay. Efforts at reversing this uncapping event by restoring the Mg^{2+} ion concentration and/or concentrating the dialysate and readdition have been unsuccessful (results not shown). The loss of actin and spectrin during dialysis (Figure 5) indicates the disintegration of the actin cytoskeleton. However, this disruption of the actin cytoskeleton occurs under very little shearing stress, suggesting structural changes in the ghost actin cytoskeleton itself. One possible explanation would be proteolysis. To prevent this, protease inhibitors were maintained in all buffers at all times and adducin (a protein sensitive to proteolysis) was monitored for signs of degradation. Proteolytic damage to adducin was minimal during the assay, suggesting that proteolysis is not responsible for the uncapping event.

This study has confirmed the capping of both ends of the actin filaments in resting human erythrocytes and highlighted the functional significance of the bivalent-cation concentration for the structural integrity of the erythrocyte cytoskeleton. A reduction in the bivalent-cation concentration leads to changes in the actin/spectrin network, reducing its strength, rendering it prone to fragmentation, especially in the absence of the plasma membrane. This effect is likely to be derived from the dissociation of spectrin from the cytoskeleton; incubation of ghosts in low-salt buffers devoid of bivalent cations is the method by which spectrin is purified [34]. This response to bivalent cations suggests a mechanism by which the physical properties of the erythrocyte can be tailored to the varying requirements during the passage through the circulatory system, as suggested by Wallis et al. [35]. During the oxygenation/deoxygenation cycle the free Mg^{2+} ion concentration oscillates between 0.6 mM and 1.9 mM [36]. It has also been demonstrated that erythrocytes put under shearing stress leak Ca^{2+} ions [37]. Hence, the oscillating Mg^{2+} ion concentration may act as a general mechanism to modulate the physical properties of the erythrocyte during the general passage through the circulatory system, supplemented by additional fine-tuning by fluctuations in the Ca^{2+} concentration during direct application of shearing stress, such as the passage through the narrow sinusoids of the spleen. Experiments to test these hypotheses are currently underway.

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REFERENCES

- Shen, B. W., Josephs, R. and Steck, T. L. (1986) Ultrastructure of the intact skeleton of the human-erythrocyte membrane. *J. Cell Biol.* **102**, 997–1006
- Byers, T. J. and Branton, D. (1985) Visualization of the protein associations in the erythrocyte-membrane skeleton. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6153–6157
- Fowler, V. M. (1996) Regulation of actin filament length in erythrocytes and striated muscle. *Curr. Opin. Cell Biol.* **8**, 86–96
- Fowler, V. M., Sussman, M. A., Miller, P. G., Flucher, B. E. and Daniels, M. P. (1993) Tropomodulin is associated with the free (pointed) ends of the thin-filaments in rat skeletal-muscle. *J. Cell Biol.* **120**, 411–420
- Weber, A., Pennise, C. R., Babcock, G. G. and Fowler, V. M. (1994) Tropomodulin caps the pointed ends of actin-filaments. *J. Cell Biol.* **127**, 1627–1635
- Gregorio, C. C., Weber, A., Bondad, M., Pennise, C. R. and Fowler, V. M. (1995) Requirement of pointed-end capping by tropomodulin to maintain actin filament length in embryonic chick cardiac myocytes. *Nature (London)* **377**, 83–86
- Kuhlman, P. A. and Fowler, V. M. (1997) 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. *Biochemistry* **36**, 13461–13472
- Kuhlman, P. A., Hughes, C. A., Bennett, V. and Fowler, V. M. (1996) A new function for adducin-calcium/calmodulin-regulated capping of the barbed ends of actin filaments. *J. Biol. Chem.* **271**, 7986–7991
- Gardner, K. and Bennett, V. (1986) A new erythrocyte membrane-associated protein with calmodulin binding-activity-identification and purification. *J. Biol. Chem.* **261**, 1339–1348
- Gardner, K. and Bennett, V. (1987) Modulation of spectrin actin assembly by erythrocyte adducin. *Nature (London)* **328**, 359–362
- Mische, S. M., Mooseker, M. S. and Morrow, J. S. (1987) Beta-spectrin bestows protein 4.1 sensitivity on spectrin–actin interactions. *J. Cell Biol.* **105**, 2837–2845
- DiNubile, M. J. (1999) Erythrocyte membrane fractions contain free barbed filament ends despite sufficient concentrations of retained capper(s) to prevent barbed end growth. *Cell Motil. Cytoskeleton* **43**, 10–22
- Hughes, C. A. and Bennett, V. (1995) Adducin—a physical model with implications for function in assembly of spectrin–actin complexes. *J. Biol. Chem.* **270**, 18990–18996
- Podolski, J. L. and Steck, T. L. (1988) Association of deoxyribonuclease-1 with the pointed ends of actin-filaments in human red blood-cell membrane skeletons. *J. Biol. Chem.* **263**, 638–645
- Pardee, J. D. and Spudis, J. A. (1982) Purification of muscle actin. *Methods Enzymol.* **85**, 164–181
- Fowler, V. M. and Taylor, D. L. (1980) Spectrin plus band 4.1 cross-link actin. Regulation by micromolar calcium. *J. Cell Biol.* **85**, 361–376
- Fowler, V. M. and Pollard, H. B. (1982) *In vitro* reconstitution of chromaffin granule–cytoskeleton interactions – ionic factors influencing the association of F-actin with purified chromaffin granule membranes. *J. Cell. Biochem.* **18**, 295–311
- Kouyama, T. and Mihashi, K. (1981) Fluorimetry study of *n*-(1-pyrenyl)iodoacetamide-labelled F-actin – local structural-change of actin protomer both on polymerization and on binding of heavy-meromyosin. *Eur. J. Biochem.* **114**, 33–38
- Weber, A., Northrop, J., Bishop, M. F., Ferrone, F. A. and Mooseker, M. S. (1987) Nucleation of actin polymerization by villin and elongation at subcritical monomer concentration. *Biochemistry* **26**, 2528–2536
- Casella, J. F., Maack, D. J. and Lin, S. (1986) Purification and initial characterization of a protein from skeletal-muscle that caps the barbed ends of actin-filaments. *J. Biol. Chem.* **261**, 10915–10921
- Caldwell, J. E., Heiss, S. G., Mermall, V. and Cooper, J. A. (1989) Effects of CapZ, an actin capping protein of muscle, on the polymerization of actin. *Biochemistry* **28**, 8506–8514
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Pinder, J. C. and Gratzer, W. B. (1982) Investigation of the actin deoxyribonuclease-I interaction using a pyrene-conjugated actin derivative. *Biochemistry* **21**, 4886–4890
- Pinder, J. C. and Gratzer, W. B. (1983) Structural and dynamic states of actin in the erythrocyte. *J. Cell Biol.* **96**, 768–775
- Tsukita, S., Tsukita, S. and Ishikawa, H. (1984) Bidirectional polymerization of G-actin on the human-erythrocyte membrane. *J. Cell Biol.* **98**, 1102–1110
- Lin, D. C. and Lin, S. (1979) Actin polymerization induced by a motility-related high-affinity cytochalasin binding complex from human erythrocyte membrane. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2345–2349

-
- 27 Brenner, S. L. and Korn, E. D. (1980) Spectrin/actin complex isolated from sheep erythrocytes accelerates actin polymerisation by simple nucleation. *J. Biol. Chem.* **255**, 1670–1676
- 28 Pinder, J. C., Ohanian, V. and Gratzer, W. B. (1984) Spectrin and protein-4.1 as an actin filament capping complex. *FEBS Lett.* **169**, 161–164
- 29 Pinder, J. C., Weeds, A. G. and Gratzer, W. B. (1986) Study of actin filament ends in the human red-cell membrane. *J. Mol. Biol.* **191**, 461–468
- 30 Pollard, T. D. and Mooseker, M. S. (1981) Direct measurement of actin polymerization rate constants by electron-microscopy of actin-filaments nucleated by isolated microvillus cores. *J. Cell Biol.* **88**, 654–659
- 31 Das, S. K. and Mukherjee, S. (1999) Heterogeneity of human red blood cell membrane: co-existence of heavy and light membranes. *Mol. Cell. Biochem.* **196**, 141–149
- 32 Matsuoka, Y., Hughes, C. A. and Bennett, V. (1996) Adducin regulation. Definition of the calmodulin-binding domain and sites of phosphorylation by protein kinase A and C.J. *Biol. Chem.* **271**, 25157–25166
- 33 Lakowicz, J. R. (1999) Instrumentation for fluorescence spectroscopy. In *Principles of Fluorescence Spectroscopy*, pp. 52–54, Kluwer Academic/Plenum Publishers, New York
- 34 Gratzer, W. B. (1982) Preparation of spectrin. *Methods Enzymol.* **85**, 475–480
- 35 Wallis, C. J., Babitch, J. A. and Wenegieme, E. F. (1993) Divalent-cation binding to erythrocyte spectrin. *Biochemistry*, **32**, 5045–5050
- 36 Bunn, H. F., Ransil, B. J. and Chao, A. (1971) The interaction between erythrocyte organic phosphates, magnesium ions, and hemoglobin. *J. Biol. Chem.* **246**, 5273–5279
- 37 Larsen, F. L., Katz, S., Roufogalis, B. D. and Brooks, D. E. (1981) Physiological shear stresses enhance the Ca^{2+} permeability of human-erythrocytes. *Nature (London)* **294**, 667–668
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