The purification of a 50 kDa protein–actin complex from unfertilized sea-urchin (*Strongylocentrotus purpuratus*) eggs

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The 100 000 g supernatant from the unfertilized egg of the sea urchin *Strongylocentrotus purpuratus* has been fractionated on DEAE-cellulose and analysed for Ca^{2+} -binding activity by the Chelex-100 competitive Ca^{2+} -binding activity assay. The major peak of Ca^{2+} -binding activity was subjected to further purification and the Ca^{2+} -binding protein responsible for this Ca^{2+} -binding-activity peak has been isolated and characterized. Non-denaturing polyacrylamide-gel electrophoresis (PAGE) followed by $^{45}\text{Ca}^{2+}$ autoradiography suggested a molecular mass of 80 kDa for the Ca^{2+} -binding protein. SDS/PAGE revealed that the 80 kDa protein consisted of a 1:1 molar complex of proteins of 50 and 42 kDa. The 42 kDa protein was identified as actin. The complex was not dissociated by extensive dialysis against an EGTA-containing buffer. The EGTA-stable complex was named '50K–A'.

INTRODUCTION

Ca^{2+} is believed to be a universal activator of sea-urchin eggs (Jaffe, 1983). A sperm initiates an increase in intracellular free Ca^{2+} when it contacts an egg at fertilization (Ridgeway *et al.*, 1976; Poenie *et al.*, 1985). Eggs can also be parthenogenetically activated by methods that increase intracellular Ca^{2+} (Chambers *et al.*, 1974; Steinhardt & Alderton, 1974; Turner *et al.*, 1986). Egg activation can be blocked by microinjecting Ca^{2+} -chelating compounds such as EGTA into eggs (Turner *et al.*, 1986; Hamaguchi & Hiramoto, 1981). Ca^{2+} appears to be required to activate many biochemical events in the sea-urchin egg.

The Ca^{2+} -dependent processes that occur after fertilization in sea-urchin eggs include: cortical-granule oocytosis (Vacquier, 1975), hardening of the fertilization membrane (Weidman *et al.*, 1985), polyphosphoinositide hydrolysis (Whitaker & Aitchison, 1985), increase in protein kinase activity (Satoh & Garbers, 1985; Ballinger *et al.*, 1984; Epel *et al.*, 1981), lipid peroxidation (Perry & Epel, 1985a,b), increase in protein and DNA synthesis (Epel, 1982; Whitaker & Steinhardt, 1985) and cytoskeletal rearrangement (Vacquier, 1981). Calmodulin, an ubiquitous Ca^{2+} -binding protein, has been identified in sea-urchin eggs (Head *et al.*, 1979) and appears to regulate several of these Ca^{2+} -dependent processes, such as cortical-granule exocytosis (Steinhardt & Alderton, 1982) and kinase activity (Epel *et al.*, 1981; Chou & Rebhun, 1986). Other Ca^{2+} -binding proteins have been identified in the sea-urchin egg. For example, Weidman *et al.* (1985) have identified a 250 kDa Ca^{2+} -binding protein called 'proteoliasin', which forms a Ca^{2+} -dependent complex with ovoperoxidase. This complex has been suggested to be an important component of the fertilization membrane. Similarly, a 45 kDa protein has been isolated from sea-urchin eggs and shown to interact with actin in a Ca^{2+} -dependent manner (Hosoya &

Mabuchi, 1984; Wang & Spudich, 1984; Coluccio *et al.*, 1986).

Previously we have shown that the Chelex-100 competitive Ca^{2+} -binding assay provides a simple method for the identification of Ca^{2+} -binding proteins in crude homogenates (Waisman & Rasmussen, 1983). In the present study we have chromatographed the 100 000 g supernatant of the unfertilized egg of *Strongylocentrotus purpuratus* on DEAE-cellulose and analysed the resultant fractions for Ca^{2+} -binding activity. The largest peak of Ca^{2+} -binding activity was subjected to further purification and the Ca^{2+} -binding protein responsible for this Ca^{2+} -binding activity peak was identified as a 50 kDa protein–actin complex ('50K–A').

EXPERIMENTAL

Materials

The buffers used in the following procedures were made with double-distilled deionized water. The chemicals used were either purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or the company listed. Also, all purification procedures were performed at 4 °C unless otherwise stated. Rabbit polyclonal antibodies to the 45 kDa protein (Wang & Spudich, 1984) were obtained from Dr. A. Spudich (Stanford University, Stanford, CA, U.S.A.).

Preparation of 100 000 g supernatants

Sea-urchins (*Strongylocentrotus purpuratus*) were collected live at Port Renfrew, British Columbia, Canada, or through the assistance of Dr. C. Glabe at the University of California at Irvine, CA, U.S.A. The animals were induced to spawn by gently removing the mouth and filling the exposed cavity with 0.5 M-KCl. The eggs were washed several times with fresh seawater and were passed through a nylon screen (0.1 mm pore size;

Abbreviations used: DTT, dithiothreitol; 50K–A, 50 kDa protein–actin complex; PAGE, polyacrylamide-gel electrophoresis; 45K–A, 45 kDa protein–actin complex.

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Tetko, New York, NY, U.S.A.) to remove debris and the jelly coat. The eggs were then either resuspended in homogenization buffer or sedimented by gentle centrifugation (1000 g) and placed into a plastic tube and stored at -80°C until required.

Fresh or frozen eggs were placed into 2 vol. of the following buffer: 40 mM-Tris (pH 7.5)/0.7 M-glucose/1 mM-DTT/1 mM-phenylmethanesulphonyl fluoride/leupeptin (0.005 mg/ml)/1 mM-benzamidine/soybean trypsin inhibitor (25 mg/l). The eggs were homogenized with either a Polytron blender (30 s bursts, ten times) for frozen eggs, or with a glass/Teflon homogenizer (five strokes over 2 min) for fresh eggs. Packed Chelex-100 resin, pH 7.5 (Bio-Rad), was stirred into the mixture to make up 5% of the total volume. The homogenate was then centrifuged at 10000 g for 30 min. The supernatant was collected and the pellet was gently resuspended in 1 vol. of buffer and re-centrifuged at 10000 g for 30 min. The two supernatants were combined and were centrifuged at 100000 g for 70 min. Occasionally a floating lipid layer, which formed in the centrifugation tube after 100000 g centrifugation, was removed and discarded. The supernatant was dialysed against 3×2 litres of 20 mM-Tris (pH 7.5)/1 mM DTT, for 18 h. The protein concentration of the non-diffusible material was as high as 4 mg/ml. The non-diffusible material was either stored at -80°C or used immediately. The colour of the 100000 g supernatants ranged from colourless (clear) to deep orange, depending upon the quantity of yolk present in the solution. Yolk was avoided because it increased the viscosity of the solution and contributed additional protein while decreasing the reproducibility of subsequent chromatographic steps. Although yolk is a major component of sea-urchin eggs, it was possible to prevent it from contaminating the 100000 g supernatant by not freezing the eggs before homogenization.

Purification of 50K-A

The dialysed 100000 g supernatant (200 ml, 4 mg/ml) was loaded on to a DEAE-cellulose (Whatman DE-52) column (2.5 cm $\times$ 20 cm) that had previously been equilibrated with 1 litre of dialysis buffer. Fractions (10 ml) were collected when the column was eluted with a linear gradient made from 400 ml each of 20 mM-Tris (pH 7.5)/1 mM-DTT and 20 mM-Tris(pH 7.5)/1 mM-DTT/0.5 M-NaCl. Portions of various fractions were dialysed before analysis by the Chelex assay. The flow-through fraction from the DEAE-cellulose column was also analysed by the Chelex assay.

The major Ca^{2+} -binding activity peak (fractions 62–82, Fig. 1 below) from the DEAE-cellulose-fractionated 100000 g supernatant was pooled and dialysed overnight against 2×1 litre of buffer A [10 mM-potassium phosphate (pH 6.8)/0.5 mM-DTT]. The dialysis residue (70 ml, 2 mg/ml) was applied to a column (2.5 cm $\times$ 10 cm) of hydroxyapatite (Bio-Rad) that had previously been equilibrated with 500 ml of buffer A. The column was eluted with a linear gradient of 10–400 mM-potassium phosphate, pH 6.8, and 0.5 mM-DTT over a volume of 400 ml (Fig. 2, below). Fractions (10 ml each) were collected and portions were analysed for Ca^{2+} -binding activity by the Chelex assay. The peak of Ca^{2+} -binding activity was pooled and concentrated by ultrafiltration (Amicon PM10 membrane) to a 2 ml volume containing 4–8 mg of protein. The concentrate was then loaded on to an h.p.l.c. gel-permeation column (TSK-3000SW;

LKB) equilibrated with 40 mM-Mops (pH 7.1)/150 mM-KCl/0.5 mM-DTT. Fractions (3 ml) were collected and were analysed for Ca^{2+} -binding activity (Fig. 3, below). The peaks of Ca^{2+} -binding activity, which consisted of 50K-A, were pooled and stored at -70°C . Occasionally minor contamination bands were present. These contaminants (< 5–10% of total protein) were removed by an additional chromatography step. The fractions were loaded on to a fast-protein-liquid-chromatography Mono-Q anion-exchange column (Pharmacia) that had been equilibrated with 10 mM-Tris (pH 7.5) buffer/0.5 mM-DTT. The column was eluted with a 0–1 M-NaCl gradient in 10 mM-Tris (pH 7.5)/0.5 mM-DTT, the purified 50K-A was monitored by SDS/PAGE (results not shown). Approx. 0.5–1.0 mg of 50K-A was purified from 100 g of eggs.

Resolution of 50 kDa protein from 50K-A

About 0.1 mg of 50K-A was mixed with SDS sample buffer, boiled, and electrophoresed into a SDS/12.5%-(w/v)-polyacrylamide gel. The gel was briefly stained and destained, rinsed in water, and then placed in a wash buffer consisting of 100 mM-Tris, pH 8.8, for 1 h. The 50 kDa protein band was cut from the gel and diced into 1–2 mm sections. An agarose tube gel was poured [2 vol. of 4% (w/v) agarose were mixed with 1 vol. of 1.5 M-Tris, pH 8.8, and 1 vol. of water] and a dialysis tube was attached to the end of the glass tube. The tube was then electrophoresed at 4 mA per tube until the Coomassie Blue dye had been eluted from the agarose. The protein was removed with a syringe and then dialysed overnight against water. The sample was then extracted with butanol to remove the Coomassie Blue dye. The aqueous phase, which contained the 50 kDa protein, was then recovered.

Transblotting and immunoblotting procedure

Transblotting and immunoblotting were performed as described by Towbin *et al.* (1979).

Non-denaturing PAGE

A 4–30%-(w/v)-polyacrylamide gradient gel was used to estimate the M_r of the 50K-A under non-denaturing conditions (Lambin & Fine, 1979). The gels were poured as a 4–30% acrylamide gradient made from a 60% acrylamide/5% bisacrylamide stock solution. The running gel buffer contained 0.375 M-Tris base, pH 8.6, and 2–15% (v/v) glycerol. The gel was polymerized with 0.015% $\text{NNN}'\text{N}'$ -tetramethylethylenediamine and 0.03% ammonium persulphate. Although a stacking gel was not used, an additional 4% acrylamide layer was poured over the running gel so that the wells of the comb would not enter into the gradient. The samples were prepared in sample buffer made without SDS or 2-mercaptoethanol and were not boiled before loading. The running buffer contained 0.089 M-Tris base (pH 8.4), 0.08 M-boric acid and 2.5 mM-EDTA. The gels were run to equilibrium (12–18 h at 375 V) while the buffer was chilled with a cooling coil. Under these conditions there was a linear relationship between the logarithm of the M_r and the logarithm of the slopes for proteins with M_r between 20000 and 660000. In all gel systems proteins were revealed by staining with 0.06% Coomassie Blue R-250 (Bio-Rad) in 10% (v/v) acetic acid and 25% (v/v) ethanol. Destaining was performed in 10% acetic acid/25% ethanol.

Analytical techniques

The protein concentration of crude and purified solutions was determined by using the Coomassie Blue dye-binding assay described by Bradford (1976). SDS/polyacrylamide gels were prepared by the method of Laemmli (1970). The Chelex-100 competitive Ca^{2+} -binding assay was performed as detailed by Tokuda *et al.* (1987). Calmodulin was assayed on the basis of its ability to activate the Ca^{2+} -activatable phosphodiesterase of bovine brain (Teo *et al.*, 1973). The elution position (K_{av}) of 50K-A from a TSK-gel G3000 SW column (Toyo Soda Co., Tokyo, Japan) equilibrated with 40 mM-Mops (pH 7.1)/150 mM-KCl/0.5 mM-DTT was determined together with those of standard proteins of known Stokes radii. The Stokes radius was determined by interpolation of the linear plot of $(-\log K_{av})^{1/2}$ versus the Stokes radius (Laurent & Killander, 1964). Anti-actin IgG was obtained from Transformation Research (Framingham, MA, U.S.A.). ^{45}Ca overlay was performed as described by Maruyama *et al.* (1984).

RESULTS

Identification of Ca^{2+} -binding activity in 100 000 g supernatants

A 100 000 g supernatant was prepared from the unfertilized eggs of the sea-urchin *Strongylocentrotus purpuratus* (see the Experimental section) and fractionated by DEAE-cellulose (Fig. 1). One major peak of Ca^{2+} -binding activity (fractions 62–82) and several smaller, poorly resolved, peaks of Ca^{2+} -binding activity were detected by the Chelex competitive Ca^{2+} -binding assay (Tokuda *et al.*, 1987). The calmodulin activity was eluted as a single peak which was resolved from the major peak of Ca^{2+} -binding activity. Ca^{2+} -binding activity could not be detected in the column flow-through. Since most of the Ca^{2+} -binding activity resolved on DEAE-cellulose could not be attributed to calmodulin, it

was reasonable to infer the presence of other, possibly novel, Ca^{2+} -binding proteins. The strategy of our subsequent experiments was therefore to purify and characterize the Ca^{2+} -binding protein(s) responsible for the Ca^{2+} -binding activity peaks. Accordingly we have purified a Ca^{2+} -binding protein complex consisting of an M_r -50 000 protein and actin (M_r 42 000) from the major peak of Ca^{2+} -binding activity. This protein complex, referred to as '50K-A', could account for the major peak of Ca^{2+} -binding activity.

Purification of 50K-A

As detailed in the Experimental section, purification of 50K-A involved several chromatographic steps, including ion-exchange on DEAE-cellulose (Fig. 1), adsorption on hydroxyapatite (Fig. 2), and gel-permeation chromatography (Fig. 3) on TSK-3000SW (LKB). Approx. 0.5–1.0 mg of 50K-A was purified from 100 g of eggs. Since the Chelex assay can only be used for qualitative analysis (Waisman & Rasmussen, 1983), we cannot estimate the amount of 50K-A present in the various fractions obtained during purification. The analysis, by SDS/PAGE, of the 50K-A-containing fractions at various steps of purification is presented in Fig. 4.

The final step in the purification of 50K-A is presented in Fig. 4. A good correlation between protein concentration and Ca^{2+} -binding activity was observed. The M_r of the Ca^{2+} -binding activity peak was estimated, by gel-permeation chromatography and assuming the complex to be spherical, to be 76 000. The Stokes radius (Laurent & Killander, 1964) of 50K-A was also estimated by this procedure to be 3.95 nm. SDS/PAGE of the peak of Ca^{2+} -binding activity resolved by gel-permeation chromatography revealed two protein bands of M_r 50 000 and M_r 42 000 (Fig. 3). Considering the Stokes radius of 2.8 nm for actin (see the Discussion below as to the identification of the M_r -42 000 protein as actin), it was reasonable to suspect the presence of a heterodimer.

Fig. 1. Chelex-assay analysis after DEAE-cellulose-chromatography of 100 000 g egg supernatants

A 100 000 g sea-urchin egg supernatant was loaded on to a DEAE-cellulose anion-exchange resin column. Proteins were eluted with a 0–500 mM-NaCl linear gradient in 20 mM-Tris (pH 7.5)/1 mM-DTT. The protein concentration was measured by the method of Bradford (1976); calmodulin was detected by the phosphodiesterase assay of Teo *et al.* (1973), and the Ca^{2+} binding activity was measured with the Chelex assay (Simpson *et al.*, 1976).

Fig. 2. Hydroxyapatite chromatography of DEAE-cellulose-chromatography fractions that contained the highest Ca^{2+} -binding activity

DEAE-cellulose-chromatography fractions that contained the peak of Ca^{2+} -binding activity (Fig. 1, fractions 62–76) were pooled and loaded on to a hydroxyapatite column. The proteins were eluted with a linear gradient of 10–300 mM-phosphate buffer, pH 6.8. The protein concentration and the Ca^{2+} -binding activity were also determined.

Fig. 3. H.p.l.c. gel filtration of the pooled Ca^{2+} -binding peak from hydroxyapatite chromatography

The peak of Ca^{2+} -binding activity from the hydroxyapatite column (Fig. 2, fractions 44–50) was pooled and loaded on to an h.p.l.c. gel-permeation column [TSK-3000SW (LKB)]. The fractions were assayed for protein concentration and for Ca^{2+} -binding. Samples of the protein peak were analysed by SDS/12.5%-PAGE (see the inset). The molecular-mass standards were lysozyme (14.4 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa) and phosphorylase (94 kDa).

In order to investigate further the possibility that the 50 kDa and 42 kDa proteins represented a protein complex, the protein was analysed by non-denaturing PAGE (Lambin & Fine, 1979). As Fig. 5 shows, a single protein band of M_r 80000 was resolved. $^{45}\text{Ca}^{2+}$ autoradiography (Maruyama *et al.*, 1984) suggested that, in

the presence of 150 mM-KCl and 5 mM- MgCl_2 , the M_r 80000 protein complex (50K-A) bound Ca^{2+} . The molar ratio of the components of this complex was estimated by densitometric scans of SDS gels to be 1:1. We have concluded that the heterodimer is a 1:1 complex of a M_r 50000 protein and a M_r 42000 protein. The amino

Table 1. Amino acid composition of the 50 kDa protein

Amino acid	Composition	
	(residues/ molecule)*†	(mol/100 mol)
Aspartic acid	28.8	5.6
Threonine	23.6§	4.6
Serine	94.6§	18.4
Glutamic acid	91.6	17.9
Proline	18.3	3.6
Glycine	102.9	20.0
Alanine	41.0	8.0
Half-cystine	ND	ND
Valine	20.2‡	3.9
Methionine	4.4	0.9
Isoleucine	11.7	2.3
Leucine	19.4	3.8
Tyrosine	5.1	1.0
Phenylalanine	10.2	2.0
Histidine	9.9	1.9
Lysine	22.2	4.3
Arginine	9.8	1.9
Tryptophan	ND	ND

* Average of values after 24, 48 and 72 h hydrolysis.

† Residues per polypeptide chain assuming an M_r of 50000.

‡ From 72 h hydrolysis only.

§ Extrapolated to zero-time hydrolysis.

|| ND, not determined.

Fig. 4. SDS/PAGE analysis at different stages of 50K-A purification

Portions of pooled fractions from each step of purification were electrophoresed into an SDS/13%-polyacrylamide gel in the following order: lane a, M_r markers; lane b, 100000 g supernatant; lane c, DEAE-cellular-chromatography peak; lane d, hydroxyapatite peak; lane e, gel-permeation peak.

Fig. 5. Non-denaturing PAGE analysis of 50K-A

Purified 50K-A was subjected to non-denaturing 4–30%-PAGE (A), followed by electrophoretic transfer to nitrocellulose and $^{45}\text{Ca}^{2+}$ autoradiography (B). M_r standards displayed in the left lane are thyroglobulin (M_r 669000), ferritin (440000), catalase (232000), aldolase (140000) and bovine serum albumin (66000).

acid composition of the 50 kDa component of 50 K-A is presented in Table 1. The protein contains about 24% Asx + Glx and 8% basic residues, and is relatively rich in glycine.

Comparison of 50K-A and 45K-A

A protein complex called '45K-A' and consisting of a 1:1 molar ratio of an M_r 45000 protein and actin (M_r 42000) has been purified from sea-urchin eggs (Wang & Spudich, 1984; Hosoya & Mabuchi, 1984; Coluccio *et al.*, 1986; Ohnuma & Mabuchi, 1986). In order to investigate potential similarities between 45K-A and the M_r 80000 protein complex, the 45K-A was purified (Wang & Spudich, 1984) and compared with the M_r 80000 proteins (Fig. 6). SDS/PAGE analysis of the 80 kDa complex and the 45K-A complex suggested that the 50 kDa component of the 80 kDa complex was significantly different in molecular size from the 45 kDa component of 45K-A. Furthermore the 45 kDa protein (Fig. 6a, lane 2) but not the 50 kDa protein (Fig. 6a, lane 3) was detected by a specific rabbit polyclonal antibody to 45K-A [obtained from Dr. A. Spudich and characterized by Wang & Spudich (1984)]. This suggested that the 50K-A complex isolated from our laboratory was distinct from 45K-A. Furthermore, the similarity in M_r between actin (Fig. 6b, lane 1) and the 42 kDa protein of the 80 kDa complex (Fig. 6b, lane 3) suggested that these proteins might be identical. As shown in Fig. 6, an anti-actin antibody cross-reacted with the 42 kDa component of the 80 kDa complex (Fig. 6c, lane 3). This result suggested that the

Fig. 6. Immunoblot analysis of 45K-A and 50K-A

Samples of rabbit skeletal-muscle actin (b, lane 1), 45K-A (b, lane 2) and 50K-A (b, lane 3) were subjected to SDS/12.5%-PAGE ('SDS/PAGE') and then transblotted on to nitrocellulose ('NC'; a and c). The nitrocellulose was incubated with anti-45K-A antibody (A) or anti-actin antibody (c) and developed (see the Experimental section).

80 kDa complex consisted of a 50000- M_r protein-actin complex. We have named this heterodimer '50K-A'. The identity of the M_r -42000 protein as actin has also been confirmed by tryptic peptide analysis (results not shown).

Separation of the 50 kDa protein from actin

We have made several attempts to separate the 50 kDa protein from actin. The complex is not separated by DEAE-cellulose, hydroxyapatite or gel-permeation chromatography. We cannot include EGTA in the original homogenization buffer to attempt to prevent formation of the heterodimer because of interference of EGTA with the Chelex assay. This interference occurs even after extensive dialysis. Furthermore, dialysis of 50K-A against 10 mM-EGTA for 10 days followed by electrophoresis on non-denaturing 4-30% polyacrylamide gels resolved a single band at 80 kDa. Although the complex could be dissociated by 6 M-urea, we found that the 50 kDa protein was irreversibly denatured by this procedure and could not associate with actin.

DISCUSSION

In the present paper we have used the Chelex-100 competitive Ca^{2+} -binding activity assay (Waisman & Rasmussen, 1983) to identify and purify a novel M_r -50000 protein that forms a 1:1 complex with actin ('50K-A'). Non-denaturing PAGE of 50K-A followed by $^{45}\text{Ca}^{2+}$ autoradiography provided additional evidence that 50K-A was a Ca^{2+} -binding-protein complex. Gershman *et al.* (1984) has suggested that incubation of G-actin in the absence of bivalent cations causes the irreversible denaturation of actin. Since measurement of Ca^{2+} -binding activity of a sample by the Chelex-100

assay requires incubation of the sample for 30 min in the absence of Mg^{2+} and in the presence of low Ca^{2+} (0.1 μM), it is not surprising that we are unable to detect Ca^{2+} -binding activity associated with G-actin. The fact that we are unable to detect with the Chelex assay Ca^{2+} -binding activity associated with G-actin suggests that the M_r -50000 protein is a Ca^{2+} -binding protein or that, after association with the M_r -50000 protein, the stability properties of actin change such that Ca^{2+} binding to actin, as detected by the Chelex assay, occurs even after incubation in the absence of bivalent cations. Clearly the question of whether or not the M_r -50000 protein of 50K-A is a Ca^{2+} -binding protein will be answered after isolation of the protein. We have unsuccessfully attempted to separate 50K-A by extensive dialysis in EGTA-containing buffers in the presence or absence of high concentrations of salts followed by non-denaturing PAGE. Although SDS/PAGE or PAGE in the presence of urea can separate the complex, the M_r -50000 protein isolated by either procedure fails to bind actin or Ca^{2+} and was therefore assumed to be irreversibly denatured.

Whether or not the M_r -50000 protein exists as a monomer *in vivo* or as 50K-A is at present unclear. Although Ca^{2+} was not added to buffers used during the isolation of 50K-A, we could not add EGTA to chelate any contaminating Ca^{2+} in these buffers because trace amounts of EGTA interfere with the Chelex assay. Therefore if 50K-A was formed during purification it probably requires only trace amounts of Ca^{2+} . The fact that 50K-A can bind Ca^{2+} suggests that the complex possesses at least one exchangeable Ca^{2+} -binding site. The observation that 50K-A is stable in EGTA suggests that the interaction of the 50 kDa protein with actin is Ca^{2+} -independent or that the complex formation is Ca^{2+} -dependent and has both exchangeable and non-

exchangeable Ca^{2+} . The presence of the exchangeable Ca^{2+} site(s) allows detection of 50K-A by the Chelex assay. In this regard it is noteworthy that in contrast with 50K-A the 45K-A complex which is formed in the presence of Ca^{2+} does not possess an exchangeable Ca^{2+} site (the dissociation half-time for Ca^{2+} was 10.8 days). One potential function of the exchangeable Ca^{2+} -binding site could be the Ca^{2+} -dependent regulation of 50K-A. This would predict that, in the presence but not in the absence of Ca^{2+} , 50K-A might bind to intracellular protein(s) from which an actin filament could nucleate. As discussed in the following paper (Golsteyn & Waisman, 1989), 50K-A shows Ca^{2+} -dependent binding to DNAase I, therefore suggesting that Ca^{2+} -dependent conformational changes occur in 50K-A. However, the effect of 50K-A on actin polymerization appears to be Ca^{2+} -independent.

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