Extracellular regulated kinase (ERK) interaction with actin and the calponin homology (CH) domain of actin-binding proteins

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An interaction between extracellular regulated kinase 1 (ERK1) and calponin has previously been reported (Menice, Hulvershorn, Adam, Wang and Morgan (1997) *J. Biol. Chem.* **272** (40), 25157–25161) and has been suggested to reflect a function of calponin as a signalling molecule. We report in this study that calponin binds to both ERK1 and ERK2 under native conditions as well as in an overlay assay. Using chymotryptic fragments of calponin, the binding site of ERK on calponin was identified as the calponin homology (CH) domain, an N-terminal region of calponin found in other actin-binding proteins. ERK also bound, in a gel overlay assay, α -actinin, a protein with two tandem CH domains, as well as a 27 kDa thermolysin product of α -actinin containing the CH domains of α -actinin. The CH domain of calponin could compete with intact calponin or α -actinin for

ERK binding. Titration of acrylodan-labelled calponin with ERK gave a K_a of $6 \times 10^6 \text{ M}^{-1}$ and titration of acrylodan-labelled calponin with a peptide from the α L16 helix of ERK gave a K_a of $1 \times 10^6 \text{ M}^{-1}$. Recombinant ERK was found to co-sediment with purified actin and induced a fluorescence change in pyrene-labelled F-actin ($K_a = 5 \times 10^6 \text{ M}^{-1}$). The interaction of ERK with CH domains points to a new potential function for CH domains. The interaction of ERK with actin raises the possibility that actin may provide a scaffold for ERK signalling complexes in both muscle and non-muscle cells.

Key words: calponin, cytoskeleton, MAP kinase, signal transduction.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases [1]. All isoforms are activated by dual phosphorylation of Thr and Tyr residues. The exact mechanism of targeting MAPK to the site of activation is unknown. Interestingly, it has been recently reported that cytochalasin disruption of actin inhibits the insulin-mediated activation of extracellular regulated kinase (ERK, p42/p44 MAPK) as well as the p38 MAPK in myotubes [2]. These findings suggest that the cytoskeleton may play a role in ERK mediated signal transduction. Evidence from our laboratory suggests that calponin may also play a role in ERK targeting [3].

Several laboratories have reported that MAPK initially undergoes a translocation from the cytosol to the plasmalemma before being activated and targeted to its substrates [4–6]. We found that in smooth muscle cells ERK is transiently targeted to the plasmalemma and co-distributed with protein kinase C (PKC) ϵ and the actin binding protein calponin at the cell surface, but then was subsequently targeted to the contractile apparatus during contraction of the cell [6]. Thus we suggested that ERK links PKC activation to the measured contractile activity. The mechanism of this targeting remains unclear, although a recent study from our laboratory reported that PKC and calponin translocate to the cell cortex simultaneously with ERK; that MAPK and PKC bind to calponin in a gel overlay assay; and, that they co-immunoprecipitate with calponin. Based on these observations, calponin was postulated to act as an adaptor molecule in ERK targeting [3]. Interestingly, in haematopoietic cells ERK is found in a complex with other proteins of which one is Vav [7–9], a protein that has homology to calponin [10].

The mechanism by which activated ERK leaves the plasmalemma is also unknown. However, the late targeting of ERK (i.e. to the contractile filaments in smooth muscle) was found to be dependent on tyrosine phosphorylation of ERK, whereas the early targeting of ERK (i.e. to the plasmalemma) occurred independently of tyrosine phosphorylation of ERK [6].

Calponin was first isolated from gizzard smooth muscle and characterized as a troponin T-like molecule that could bind actin and calmodulin [11]. Subsequent imaging studies have cast doubt on calponin's role as a physiological inhibitor of actomyosin ATPase activity since calponin was localized with non-contractile β -actin and desmin to a greater extent than with contractile actin [12,13]. Data from our laboratory indicate that in the contracted state this is indeed the case, but that in the relaxed state, calponin is associated with contractile α -actin [14,15]. Thus, at the present time, it remains controversial as to whether calponin is a contractile regulatory protein [16] or a regulator of signal transduction [3].

We present evidence in this report that the calponin homology (CH) domain of calponin is the region which interacts with ERK, providing a new potential function for the CH domain. Evidence is also presented that ERK can directly bind actin, opening the possibility that actin could provide a scaffold for ERK signalling complexes in both muscle and non-muscle cells.

MATERIALS AND METHODS

Preparation of proteins

Calponin was prepared from turkey gizzard [17]. α -Actinin was prepared from turkey gizzard [18]. Actin was a gift from Dr.

Abbreviations used: CH, calponin homology; ERK, extracellular regulated kinase; ERK2P₂, diphosphorylated p42 ERK; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NEPHGE, non-equilibrium pH gradient electrophoresis; PKC, protein kinase C.

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Terrance Tao. Tropomyosin was a gift from Dr. Sherwin Lehrer. Myosin was a gift from Dr. Joseph Chalovich. Grb was a gift from Dr. Celia Harrison. ERK1 was prepared as described previously [19]. ERK2 was coexpressed with constitutively active MAPK kinase (MEK) using a construct generously provided by Dr. Melanie Cobb [20]. Thermolysin and α -chymotrypsin were purchased from Sigma.

Overlay assay

Blots blocked with 5% milk were incubated with 10 μ g/ml diphosphorylated p42 ERK (ERK2P₂) expressed in *Escherichia coli* or 50 μ g/ml calponin isolated from turkey gizzard for 1 h in a solution containing 10 mg/ml non-fat milk and protease inhibitors as previously reported [3] in 150 mM NaCl and 10 mM Tris or imidazole, pH 7. Blots were fixed in 0.5% formaldehyde, neutralized in 2% glycine, rinsed with phosphate buffered saline, and processed with antibodies as for Western blots.

Calponin and α -actinin proteolysis

Calponin was digested with α -chymotrypsin as previously described [21]. Basically, a 1–2 mg/ml solution of turkey gizzard calponin was digested with α -chymotrypsin at a w/w ratio of 1:1000 (limited digest) or 1:100 (extended digest) for 1–15 min (limited digest) or for 30 min (extended digest) at room temperature. An additional digestion was performed with a chymotrypsin/calponin ratio of 1:100 for 1 h, which we have termed an 'exhaustive' digest. Chymotryptic peptides were separated by SDS/PAGE and electroblotted on to Immobilon-P membranes (Millipore) using a CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) buffer system [22]. Their identities were confirmed by N-terminal sequence analysis using an Applied Biosystems Model 477A sequencer. α -Actinin was digested with thermolysin [23] using a 1:25 w/w ratio of thermolysin to α -actinin for 1–2 h and the 27 kDa fragment was confirmed to have the same N-terminal sequence as the 27 kDa, thermolysin cleaved actin binding domain from chicken gizzard [24].

Nickel bead sedimentation

Nickel beads (NTA resin, Qiagen Inc., Chatsworth, CA, U.S.A.), 10 μ l, were aliquoted from a 10% packed stock. Histidine tagged proteins were added directly to beads and allowed to incubate at room temperature for 5 min. Beads were sedimented by microcentrifugation for approx. 5 s so as not to damage the beads. The solution composition was 20 mM Tris/HCl pH 7.4, 50 mM NaCl, 0.5 mM EGTA, and 5 mM β -mercaptoethanol. The supernatant was removed and calponin and buffer were added to the beads so as to make the final concentration of ERK 5 μ M and 30 μ M calponin in 60 μ l of solution. Beads were microcentrifuged; the supernatant containing calponin was collected. Beads were rinsed with 100 μ l of buffer. After a brief microcentrifugation, the supernatant was collected and precipitated with 5% trichloroacetic acid (TCA). The beads were then rinsed with 100 μ l of 1 M imidazole pH 7.0 to remove calponin bound to ERK or Grb.

Non-equilibrium isoelectric focusing gels

Bio-Rad 1 mm thick minigels were used to separate isoelectric variants of calponin. The gel composition was essentially the same as previously described [25]. The cathode solution was 20 mM NaOH while the anode solution was 20 mM H₃PO₄. His-tagged proteins were sedimented with nickel beads. In an

ticipation of low protein affinities, the proteins on the beads were washed only once, with $\times 20$ the volume. Proteins were eluted from the beads with imidazole. Protein was loaded on the anode side. Focusing was carried out at 500 V for 45 min. After focusing, the gel was transferred to Immobilon-P using semi-dry electroblotting according to the protocol supplied by Hoeffer Scientific Instruments (San Francisco, CA, U.S.A.) with one modification. The cathode filter directly in contact with the gel was soaked in 30 mg of SDS dissolved in the minimal amount of buffer needed to saturate the filter. After transfer, blots were blocked with 5% milk and stained with a 1:5000 dilution of polyclonal antibody against calponin, which was a gift from Dr. Katsuhide Mabuchi (Boston Biomedical Research Institute, Boston, MA, U.S.A.).

Tissue preparation

Aorta whole cell homogenate was prepared from previously frozen ferret aorta [3] using isoelectric focusing lysis buffer [25] because this buffer is very efficient in solubilizing all of the tissue. Solubilized aorta was diluted 1:1 with SDS/PAGE sample buffer for overlays. Ferrets were anaesthetized with chloroform in a ventilation hood, and the aorta was removed to an oxygenated physiological saline solution (PSS). The endothelium was removed from the aorta before being processed.

High-speed sedimentation assay

Sedimentation of recombinant ERK2 with rabbit skeletal muscle actin was performed in solution A: 150 mM NaCl, 10 mM imidazole pH 7.0, 2 mM MgCl₂, 0.5 mM EGTA, 0.01% NaN₃, and 15 mM β -mercaptoethanol. The concentration of actin used was 5 μ M and ERK was 0.5 μ M. Sedimentation was at 100000 g for 30 min at 20 °C. Because of the limited ability of SDS/PAGE to resolve ERK2 from actin, Western blot analysis was used to confirm the presence of ERK in the pellet.

Fluorescence labelling of calponin and actin

Calponin was labelled with acrylodan as previously described [27,28]. The extent of acrylodan incorporation was determined using an absorption coefficient of 16400 M⁻¹·cm⁻¹ at 387 nm. Rabbit skeletal muscle actin was labelled with pyrene iodoacetamide [29]. The extent of pyrene incorporation was determined using an absorption coefficient of 2.2 $\times 10^4$ M⁻¹·cm⁻¹ at 344 nm. The concentrations of actin and calponin were determined by a modified Lowry assay (Bio-Rad, Hercules, CA, U.S.A.) using BSA as a standard.

Fluorescence titrations

All titrations were performed at room temperature (20–22 °C) in solution A with 20 mM imidazole using a Spex Fluorolog spectrofluorimeter. Absorption at the wavelength of excitation never exceeded 0.1, so no correction was made for the inner filter effect. Pyrenyl actin, 0.5 μ M, was excited at 344 nm and emission was monitored from 340 to 440 nm. Acrylodan-calponin, 0.2–1 μ M, was excited at 387 nm and the emission was monitored from 380 to 600 nm.

Analysis of fluorescence data

The affinity of ERK and the ERK peptide α L16 for calponin was determined by fitting the fluorescence signal as function of protein concentration. The fluorescence change was taken to be proportional to the fractional saturation of binding sites (ν),

which was determined by calculating the quadratic root of the following equation:

$$nav^2 - (K_d + na + b)v + b = 0,$$

where n is the apparent stoichiometry and a and b are the total concentrations of calponin and ERK, respectively, K_d is the dissociation constant. The best fit was obtained when the stoichiometry was assumed to be 1:1.

The affinity of ERK for pyrenyl actin was determined from the average fluorescence between 518 and 522 nm corrected for dilution. The affinity of ERK for actin was estimated using data obtained from both the first and second pyrene emission peaks. Binding was assumed to be 1:1 and it was assumed that binding of ERK to each actin subunit occurred independently. n was fixed at 1.0 during the fitting.

RESULTS

Calponin binds to ERK1, ERK2 and ERK2P₂ immobilized on PVDF

It has previously been demonstrated that ERK1 binds to membrane bound gizzard (basic) calponin in the gel overlay [3].

Figure 1 Calponin binds ERK in the overlay assay

(A) Overlay assay. Whole cell homogenate (lane 1), gizzard α -actinin (lane 2), calmodulin (lane 3), α - and β -smooth muscle tropomyosin (lane 4), gizzard smooth muscle myosin (lane 5), rabbit skeletal muscle actin (lane 6), recombinant ERK1 (lane 7), and bovine serum albumin (lane 8) were separated by SDS/PAGE, transferred to Immobilon membrane, incubated with calponin then incubated with calponin antibody to determine which proteins bound calponin (right panel). (B) Untreated and acid phosphatase treated expressed diphosphorylated ERK2 were immunoblotted with ERK2 antibody (left panel) or antibody specific for diphosphorylated ERK (right panel). (C) Left panel: Approximately equal-molar amounts of unphosphorylated ERK1 and diphosphorylated ERK2 were incubated with calponin and then immunoblotted with antibody against calponin. Right panel: Diphosphorylated ERK2 was subjected to partial dephosphorylation with acid phosphatase, incubated with calponin and then immunoblotted with antibody against calponin. Similar results were obtained for alkaline phosphatase treated ERK2P₂. These results are typical of 2–5 experiments.

Since the membrane bound proteins may be denatured we first asked the question of whether calponin in solution would also bind to ERK1 immobilized on an Immobilon P membrane. Calponin indeed does bind immobilized ERK1 (Figure 1, lane 7). Note that recombinant ERK1 runs as a doublet. Both bands of ERK react with ERK antibody but do not react with antibody raised against phosphorylated ERK. Thus, the second band is likely due to limited proteolysis. Calponin also binds actin (lane 6), a well characterized binding partner [26]. In the ferret aorta whole cell homogenate (lane 1), bands at ~ 45 kDa (likely actin and ERK), as well as ~ 34 kDa (likely calponin), also gave strong staining. However, it should be noted that calponin, a highly basic protein, also binds bovine serum albumin (lane 8) raising the question of non-specific interactions. In contrast, calponin did not detectably interact with gizzard smooth muscle myosin (lane 5), smooth muscle tropomyosin (lane 4) or calmodulin (lane 3), although these proteins have been described as low affinity binding partners of calponin as has been previously reviewed [26]. Thus, although calponin may bind some proteins non-specifically, calponin shows preference for ERK1.

Calponin was found to also bind phosphorylated ERK2 coexpressed in *E. coli* with constitutively active MEK [20] (Figure 1C). The question arose as to whether phosphorylation has any effect on binding. Thus, ERK2P₂ was subjected to potato acid phosphatase digestion. By analysis with an antibody specific for diphosphorylated ERK1 and ERK2 (Figure 1B, right panel) in comparison with an antibody to all ERK2 (Figure 1B, left panel), it was confirmed that significant amounts of the ERK2P₂ were dephosphorylated. In the overlay assay, calponin showed little or no preference for the ERK1 isoform of MAPK compared with the phosphorylated ERK2 isoform (Figure 1C, left panel), nor did dephosphorylation of ERK2 result in a dramatic change in affinity for calponin (Figure 1C, right panel). There may be some preference of the unphosphorylated ERK2 for calponin, but both phosphorylated and unphosphorylated ERK2 bound calponin. Based on these results it was concluded that ERK2P₂ behaved quite similarly to ERK1 in regard to calponin binding and ERK2P₂ was used throughout this study because it was more readily available.

Calponin binds ERK on nickel beads under native conditions

Because the gel overlay assay has a tendency for false-positives as well as false-negatives, and because proteins on the membrane may not be properly folded, it was of interest to investigate the ERK/calponin interaction under native conditions. Thus we attempted to co-sediment histidine tagged ERK2P₂ and calponin using nickel (NTA) agarose beads. As a control, calponin binding to nickel beads alone and nickel beads to which the protein Grb was attached via a polyhistidine tag were also used. Native gizzard (basic) calponin is composed of several isoelectric variants whose origin appears to be post-translational modification other than phosphorylation [30–32]. We investigated the possibility that one of these uncharacterized post-translational modifications might regulate ERK binding to calponin. Non-equilibrium isoelectric mini gels were used to resolve these isoelectric variants. We found that significantly more calponin bound to the ERK beads than to the Grb beads and that more than five bands (isoelectric variants) of calponin were resolved from the ERK pellet. After normalization for protein content, we were unable to see any obvious preference of any of the bands for binding to the ERK beads (data not shown). Because there was no obvious difference amongst the isoelectric variants, we used native calponin for the remainder of the study.

Figure 2 Calponin labelled with acrylodan to detect binding to ERK

(A) 0.5 μM acrylodan calponin alone (solid line) was titrated with ERK causing a decrease in fluorescence intensity. Shown here is 0.2 μM (dashed line) and 1.0 μM (dotted line). These data are representative of one of three separate titrations. (B) Titration data were fit to obtain an affinity of $3.8 \times 10^6 \text{ M}^{-1}$ as described in the Materials and methods section. (C) When 1 μM acrylodan calponin (solid line) is titrated with αL16 (1 μM dashed line, 20 μM dotted line), a fluorescence decrease is seen. (D) Titration data were fit to obtain an affinity of $1.0 \times 10^6 \text{ M}^{-1}$ as described in the Materials and methods section.

Acrylodan-labelled calponin binds ERK and an ERK peptide with high affinity

Using a previously described method, we were able to achieve 90% label incorporation, presumably at Cys273 as has been previously described for this reaction [28]. As a positive control, the addition of calcium and calmodulin resulted in an increase in fluorescence and a 20–30 nm blue shift in the maximum wavelength of fluorescence as previously described [27] that could be reversed by a molar excess of unlabelled calponin. Titration of 0.2 μM acrylodan calponin with up to 20 μM bovine serum albumin as a negative control resulted in only a minor decrease in fluorescence intensity (data not shown). A fluorescence decrease was seen when acrylodan-labelled calponin was titrated with ERK (Figure 2A). Three separate titrations of acrylodan calponin with ERK (one of which is shown in Figure 2B) revealed a mean affinity of $6.4 \pm 1.5 \times 10^6 \text{ M}^{-1}$.

We also looked at the interaction between labelled calponin and a peptide from a domain of ERK that we suspected might be involved in calponin binding. The αL16 helix of ERK is known to be involved in dimerization of ERK seen *in vitro* in glycerol-containing buffers and should not be available for binding after dimerization [33]. Cellular studies have shown that calponin co-localizes with ERK before, but not after the phosphorylation-induced dimerization of ERK [6,34]. Thus, we postulated that the αL16 helix might be involved in the CaP binding site. Acrylodan-calponin was titrated with the αL16 peptide (Figure 2C). The affinity determined by two separate titrations was 0.95 and $1.3 \times 10^6 \text{ M}^{-1}$ (Figure 2D). Thus, the αL16 region of ERK has the potential to be one of the regions which interacts with calponin.

Figure 3 Mapping of the ERK binding domain on calponin

(A) Calponin was digested with α -chymotrypsin [21]. For a limited digest the ratio of α -chymotrypsin to calponin was 1:1000, w/w. Incubation times were 1–15 min. Left panel: diagram of cleavage sites elucidated by N-terminal sequencing of bands at 31, 22 and 14 kDa shown in the middle panel. ERK2, 10 mg/ml, was used to overlay the digest products, right panel. (B) Calponin was also digested for 1 h with a chymotrypsin to calponin ratio of 1:100, w/w. N-terminal sequencing revealed that the three major bands shown in the middle panel were all derived from the N-terminal half of the calponin molecule. ERK2 overlay revealed that all three N-terminal fragments of calponin bound ERK, right panel. These results are typical of three experiments.

ERK binds the CH domain of calponin

We next asked which region of calponin binds to ERK. To accomplish this, calponin was subjected to a limited and an extended digestion with chymotrypsin [21] (Figures 3A and 3B, middle panel). N-terminal sequencing was used to confirm the identity of the fragments shown diagrammatically in Figures 3A and 3B, left panels. For the limited digest (Figure 3A, left panel), the first five N-terminal residues for the 22 kDa fragment were NRGPA, and for the 14 kDa fragment were GTRRH. The 31 kDa fragment was N-terminally blocked, indicating that it originated from calponin cleaved at the C-terminal end. These chymotryptic fragments were used for the membrane bound phase of the overlay assay while ERK2P₂ was used in the solution phase. For the limited digest (Figure 3A, right panel), robust ERK binding was detected to full length calponin, the large 31 kDa fragments, and the 22 kDa fragment containing both the actin binding TnI-like region and the CH domain. Little or no binding was seen with the C-terminal 14 kDa fragment. Similar results were obtained using ERK1 (data not shown). Thus, ERK binding is specific for the N-terminal part of calponin.

To further define the binding domain, we also used an extended digest, which has been shown [21], and we have confirmed, to be composed of: the 22 kDa fragment (also present in the limited digest and which contains the TnI-like region and the CH domain), a band of approx. 16 kDa, and another band of approx. 14 kDa. N-terminal sequencing was performed and all three fragments were found to begin with the sequence NRGPA. This, plus the approximate molecular weight based on gel migration, indicates that the 14 kDa fragment contains only the CH domain while the 16 kDa fragment may contain a small fraction of the actin binding domains as well (Figure 3B, left panel). ERK binds to all three extended digest fragments with about equal intensity (right-hand side of Figure 3B). Diminished binding of ERK to proteolytic fragments of calponin compared with whole calponin (right-hand side of Figure 3A) could be due to a direct or indirect contribution of the other domains of

Figure 4 ERK2 binds to the CH domain of α -actinin

(A) α -Actinin is a protein which contains two tandem N-terminal CH domains. ERK2 binds purified α -actinin in an overlay assay. (B) α -Actinin was digested for the indicated times with thermolysin [39]. ERK2 binds very strongly to the 27 kDa fragment previously described as the N-terminal actin binding region (CH domains), solid arrow. Very little binding to the spectrin-like repeats is seen at 54 kDa, hollow arrow. (Binding to α -actinin was repeated at least five times. Binding to the 27 kDa fragment was repeated three times.)

Figure 5 The CH domain of calponin prevents ERK2 from binding to calponin, actin, and α -actinin

As confirmation of the ability of the CH domain of calponin to bind ERK under native conditions, an exhaustive digest was prepared as shown in (A) lane 2. Lane 1 shows the molecular weight markers used. It was found by mass spectrum analysis that a 3.1 kDa peptide derived from the non-ERK binding C-terminus based on molecular weight was hidden in the dye front. This is not anticipated to confound the results. (B) When approx. 30 μ M of this digest was added with 0.14 μ M ERK2 in the overlay assay, ERK2 binding to calponin, actin and α -actinin was almost completely abolished (middle) compared with the control (left). Full-length calponin, right panel, also inhibited binding of ERK to immobilized calponin, actin, and α -actinin. (Figure is representative of three separate experiments.)

calponin to ERK binding. It was, therefore, desirable to demonstrate an ERK interaction with a CH domain of a protein other than calponin.

The CH domain region of α -actinin binds ERK

The CH domain is found in many actin-binding proteins as well as signalling proteins. It was therefore of interest to determine if ERK interacts with other CH domain proteins. α -Actinin is another such CH domain-containing protein. In overlay assays, ERK was found to bind to purified gizzard α -actinin (Figure 4A) as well as a band of about 100 kDa in whole cell homogenates that migrates with α -actinin (data not shown). Thermolysin

digestion has previously been used to obtain a 27 kDa α -actinin fragment containing the CH domains and an $\sim$ 54 kDa domain containing the spectrin like repeats [23,24,35,36] (Figure 4B, left panel). The N-terminal sequence of the 27 kDa fragment was LLDPA, indicating that the first 24 amino acids of the N-terminus had been cleaved. These results are the same as those obtained by Xu and colleagues [24] who confirmed presence of the CH domains via mass spectrum analysis. When this digest was used in the membrane phase of an ERK2 overlay, binding to the 27 kDa fragment (Figure 4, filled arrow) was much greater than that to the 54 kDa fragment containing spectrin repeats (Figure 4, open arrow).

The CH domain competes with ERK binding to calponin and α -actinin

Finally, if the CH domain of calponin binds ERK under native conditions, calponin as well as the CH domain of calponin in solution should inhibit ERK binding to membrane bound calponin in the gel overlay assay. To test this hypothesis, an 'exhaustive' (one leaving only the CH domain) digest of calponin was prepared (Figure 5A). The prominent band just under 14 kDa was determined by mass spectrometry to have an actual mass of 8.4 kDa and by N-terminal sequencing to start with the sequence NRGPA. Thus, based on the known sequence of gizzard calponin, it contains amino acids 7–91, which include CH domain helices A–D, some of helix E, but not helices F or G. The digest was used to compete with ERK for membrane-bound calponin in the overlay assay (Figure 5B). The concentration of ERK in the overlay solution was 6 μ g/ml (0.14 μ M). Approx. 1.2 mg/ml (35 μ M) of the 1 h exhaustive digest and the same amount of undigested calponin were used to compete with ERK. As can be seen (middle panel, Figure 5B), the ERK binding to calponin in the presence of CH domain is less than in its absence (left panel, Figure 5B). This is further evidence that an interaction between ERK and the CH domain can occur under native conditions. The CH domain of calponin blocked binding of ERK to α -actinin (Figure 5B, left versus middle panel). Similar results were obtained with whole calponin (right panel Figure 5B).

The exhaustive digest also contained a 3.1 kDa fragment (determined by mass spectrometry) that ran with the dye front (Figure 5A). This fragment did not bind ERK. Its molecular weight determined by mass spectrometry and the known chymotrypsin digestion sites places it at amino acids 261–290, consistent with the fact that the C-terminal fragment from the limited digest did not bind ERK. Thus, we did not further pursue its characterization.

ERK binds actin

We noted in the overlay assay shown in Figure 5B, that ERK binds to actin and also that both full-length calponin and the calponin CH domain inhibit ERK binding to actin. Although whole calponin may be competing with ERK for the same binding site on actin, the CH domain does not bind actin. Therefore, the binding of the CH domain to ERK is somehow blocking the interaction of ERK with actin either by blocking the actin binding site on ERK or by inducing a conformational change in ERK. Interaction of actin with ERK was also suggested by our earlier work in which actin immunoprecipitated with ERK [3]. The interpretation of these results was unclear, however, since actin is a major smooth muscle protein and may have been a contaminant in the immunoprecipitate.

The possibility of an ERK–actin interaction was of interest, since in deconvoluted high resolution fluorescent images, acti-

Figure 6 Purified ERK2P₂ binds actin

ERK2P₂, 0.5 µM, is able to sediment in the presence of 5 µM F-actin at 100 000 *g* (right panels), but not in its absence (middle panels). S, supernatant, P, pellet. Western blot analysis was used to confirm that the bands beneath actin are ERK. These results are representative of five separate experiments.

vated ERK co-localized with filamentous bundles in freshly isolated ferret aorta cells activated with phenylephrine [6]. It was of interest, therefore, to determine if ERK binds actin under native conditions. Figure 6 shows that actin alone, but not ERK alone, comes down in the pellet in a sedimentation assay, and that ERK co-sediments with F-actin, as confirmed by Western blot analysis. The amount of ERK sedimenting with actin was variable and seemed to be dependent on time since dialysis out of the storage buffer described previously [20].

Since ERK and actin migrate at the same position in SDS/PAGE gels and gel densitometry, we were unable to determine the affinity by these techniques. Thus we used pyrenyl labelled actin to measure the affinity. Figure 7A shows changes in the

pyrenyl actin emission spectra with the addition of ERK. High-speed sedimentation confirmed that > 90% of actin was polymerized at the same concentration used for fluorescence measurements. Figure 7B shows a binding curve using the assumptions detailed in the methods. The ERK actin affinity is approx. 5×10^6 M⁻¹.

DISCUSSION

One of the main findings of the present study was that ERK binds the calponin homology domain. The protein sequence of the actin binding protein has been divided into three domains. The middle domain of calponin has homology to TnI [37] and it has been shown to mediate actin binding in the basic (smooth muscle) isoform of calponin. The C-terminal region of calponin is composed of three highly conserved repeats. The repeating sequences have recently been described as a second actin binding domain [38,39]. The most N-terminal domain has been named the calponin homology or CH domain. Two CH domains in tandem constitute the actin-binding domain of numerous cytoskeletal proteins such as α -actinin, spectrin, dystrophin and fimbrin, which has four CH domains. Single CH domains have been found in small GTPase protein binding partners Vav and IQGAP [40]. The single CH domain of calponin is neither necessary nor sufficient for calponin's actin binding [38] but can mediate actin co-localization by the signal transduction protein IQGAP [41] as well as actin binding [42], and has been suggested to be necessary for Vav targeting to actin [10].

We have shown that ERK interacts with a fragment of calponin produced by an extended digest with chymotrypsin that runs at 13–14 kDa on SDS/PAGE gels. N-terminal sequencing confirmed that this region contains the CH domain of calponin. We have also shown that ERK interacts with α -actinin in an overlay assay. α -Actinin contains two tandem CH domains at its N-terminus. Thermolysin cleavage of α -actinin yields a 27 kDa fragment to which ERK was also able to bind. The CH domain produced by an exhaustive digestion of calponin with chymotrypsin was able to prevent ERK from binding to the Immobilon membrane-bound α -actinin, further confirming the role of this

Figure 7 Interaction between ERK and pyrenyl actin monitored by fluorescence emission

(A) 0.5 µM pyrenyl actin was titrated with activated ERK (ERK2P₂). Excitation was at 344 nm. For clarity only four traces are shown; the buffer composition was the same as that in Figure 5, i.e. no ATP was in the mixture. At least 90% of the actin was polymerized as judged by high-speed sedimentation. (B) Fluorescence changes at 386–389 nm (first pyrene peak) and 405–408 nm (second pyrene peak) were averaged. The fitting gives an affinity of 5.4×10^6 M⁻¹, $R = 0.973$.

domain in interaction with ERK. Thus, although the CH domain of calponin was first proposed to function as an actin-binding domain, the current study suggests that it may have an important role in interacting with signalling molecules such as ERK.

A second major finding of the present study was that purified, diphosphorylated (activated) ERK2 can bind to purified rabbit skeletal muscle actin under native conditions. This is consistent with a previous observation from this laboratory that ERK translocates from the cell cortex to the region of the thin filaments upon phosphorylation. At this point the translocation of ERK to an actin-rich region seems unique to differentiated smooth muscle. In contrast, most investigators have reported translocation of ERK to the nucleus upon activation of cultured cells. The content of actin in smooth muscle, however, is quite high compared with non-muscle cells and cultured smooth muscle cells. Given an actin concentration of $870 \mu\text{M}$ [43] and an affinity of $5 \times 10^6 \text{ M}^{-1}$, virtually all of ERK could be associated with actin even if only 1% of the sites are available. Where and when ERK associates with actin is likely to depend on competition as well as synergism with other actin-binding proteins.

In view of the interactions determined in this work, the question arises as to whether ERK will interact with a CH domain protein in the cell. We do not have sufficient *in vitro* data to model all of the *in vivo* interactions between these proteins. Using fluorescence techniques, we report here that ERK has similar affinities for calponin and actin. The concentration of total actin in the cell is perhaps $\times 30$ greater than that of calponin [43] and there are undoubtedly other factors involved. Previous imaging results (at a level of resolution of better than 140 nm, achieved by mathematical deconvolution of digital images) from this laboratory have shown that calponin co-localizes with ERK but not with actin at early time points, but that ERK co-localizes with actin but not with calponin at late time points after agonist activation [3,6,14,15,34]. These cellular results combined with the *in vitro* results presented here are certainly consistent with the possibility that ERK interacts directly with both calponin and actin under appropriate conditions of cellular stimulation.

In summary, we have shown that ERK interacts with the CH domain of both calponin and α -actinin and that recombinant ERK2P₂ can bind directly to actin. Our findings that ERK cannot only associate with actin but also with CH domains which make up actin-binding domains of a vast array of cytoskeletal proteins as well as the signalling proteins IQGAP and Vav, point to a possible interaction between ERK-dependent signalling and cytoskeletal dynamics.

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