Actin Filament Cross-Linking by Chicken Gizzard Filamin Is Regulated by Phosphorylation *in Vitro*†

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ABSTRACT: Filamin is a dimeric muscle phosphoprotein that cross-links actin filaments. We have found that purified chicken gizzard filamin is phosphorylated *in vitro* at serine residues by the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Up to 0.9 mol of phosphate can be incorporated into 1 mol of filamin dimer. Phosphorylation by CaM kinase II increases filamin's critical actin filament gelling concentration and diminishes the amount of actin sedimented by filamin at low G-force. The modulation of filamin function by CaM kinase II requires ATP, Ca^{2+}, and calmodulin, and it is abolished when CaM kinase II is inactivated with heat. Protein phosphatase 2A removed the phosphate added by CaM kinase II and restored filamin's actin filament cross-linking activity to the untreated basal level. In cosedimentation experiments, phosphorylation reduces the binding of filamin to actin filaments. The K_d for binding of filamin to actin filaments increases ~2-fold, from 3.2 to 6.9 μM, following CaM kinase II-mediated phosphorylation. Phosphorylation by CaM kinase II, therefore, regulates the binding of filamin to actin filaments.

Filamin and ABP-280[1] are members of a homologous protein family that share a 560-kDa molecular mass, a cortical localization in cells, and most importantly the ability to cross-link actin filaments into three-dimensional networks (Hartwig & Kwiatkowski, 1991). Both are elongated dimers composed of identical subunits, each composed predominately of a long rod domain of β-sheet repetitive motifs that connects an amino-terminal actin-binding domain to a carboxyl-terminal self-association site (Gorlin et al., 1990; Hock et al., 1990). Since each molecule has two actin-binding sites, both proteins have the capacity to cross-link actin filaments; when mixed with actin, filamin and ABP-280 can gel a solution of actin filaments (Brotschi et al., 1978).

Although highly related in structure, ABP-280 is ~5-fold more potent than chicken gizzard smooth muscle filamin in its ability to cross-link actin filaments into a gel (Brotschi et al., 1978). This may be due to differences in the primary structure of the two molecules. The chicken filamin isoform obtained from Muller glial cells encodes a polypeptide chain with a gap of 23 amino acids found between repeats 15 and 16 of nonmuscle ABP-280 (Barry et al., 1993). This region in the nonmuscle isoform of ABP-280 encodes a hinge postulated to be important for molecular flexibility of the actin-binding site and which contains a calpain cleavage site. This calpain cleavage site is thus absent from the chicken protein (Gorlin et al., 1990; Barry et al., 1993). Interestingly, in human tissue, the skeletal and cardiac muscle isoforms of ABP-280 also lack the hinge structure and are products of chromosome 7, while nonmuscle ABP is on chromosome X (Gorlin et al., 1993; Maestrini, et al., 1993). Multiple isoforms of chicken filamin have also been observed, but their differences at the level of primary structures are unclear (Pavalko et al., 1989; Lemmon, 1986; Gomer & Lazarides, 1983a,b).

Filamin and ABP-280 are known to be phosphorylated in cells (Sefton et al., 1981; Carroll & Gerrard, 1982) and cell lysates (Davies et al., 1977; Wallach et al., 1978). However, the functional importance of the phosphorylation remains unclear. Both cAMP-dependent protein kinase (Wallach et al., 1978; Chen & Stracher, 1989) and protein kinase C (Kawamoto & Hidaka, 1984) phosphorylate filamin and ABP-280 *in vitro*. Two structural effects of cAMP-mediated phosphorylation have been detected. First, ABP-280 phosphorylated by cAMP-dependent protein kinase is more resistant to the protease calpain (Chen & Stracher, 1989). Second, the interaction of ABP-280 with a GTP or GTP-binding protein is altered by phosphorylation (Yada et al., 1990; Ueda et al., 1992). The effects of phosphorylation on filamin's actin cross-linking activity remain unexplored. We found chicken gizzard filamin to be a substrate for the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) *in vitro*. Addition of 1 mol of phosphate per dimer by CaM kinase II modulates filamin's actin filament cross-linking activity.

EXPERIMENTAL PROCEDURES

Reagents. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{32}P]orthophosphate (8500−9120 Ci/mmol) were purchased from DuPont-New England Nuclear Corp. Trypsin, chymotrypsin,

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[1] Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; K_d, apparent dissociation constant; ABP, actin-binding protein.

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calpain, phenylmethanesulfonyl fluoride (PMSF), β-amylase, thyroglobulin, and polyclonal goat anti-chicken gizzard filamin antibody were purchased from Sigma. XAR-5 X-ray film was from Eastman Kodak. Calmodulin and CaM kinase II were purified from porcine and rat brain, respectively, as previously described (Ohta et al., 1988). Actin was purified from rabbit skeletal muscle (Spudich & Watt, 1971). In some experiments, actin was further purified by additional depolymerization−repolymerization cycles (Pardee & Spudich, 1982). Chicken gizzard filamin was purified according to Feramisco and Burridge (Feramisco & Burridge, 1980). The catalytic subunit of protein phosphatase 2A was prepared according to the method of Cohen (Cohen et al., 1988).

Phosphorylation Assays. Phosphorylation assays were carried out at 30 °C with or without Ca^{2+}, calmodulin, and ATP. The standard reaction volume of 50 μL contained 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 10 mM $MgCl_2$, 0.3 mM EGTA, 1.0 mM $CaCl_2$, 3 μM calmodulin, and 0.5 mM [γ-^{32}P]ATP. To determine the kinetics and stoichiometry of filamin phosphorylation, the phosphorylation reaction was stopped at various times by adding SDS−PAGE sample buffer, boiled for 2 min, and displayed by SDS−PAGE according to the method of Laemmli (Laemmli, 1970). SDS−PAGE gels were stained with Coomassie brilliant blue, dried, and autoradiographed. In some experiments, the Coomassie-stained filamin subunit was excised and the ^{32}P content was determined by liquid scintillation counting.

Phosphorylation of Filamin in Intact Chicken Gizzard. Tissue filamin was phosphorylated *in vivo* according to the method of Wallach et al. (Wallach, 1978) with some modifications. Fresh gizzards were collected from chickens killed by decapitation in phosphate-free Tyrode buffer containing penicillin G (100 units/mL) and streptomycin (100 μg/mL). The gizzard tissue was sliced with a razer blade into small pieces (about 0.2 g), which were transferred into 35-mm cell culture dishes containing 2 mL of phosphate-free Tyrode buffer and incubated for 15 min in a CO_2 incubator (37 °C with 5% CO_2). [^{32}P]Orthophosphate (0.25 mCi/mL) was added to the petri dishes, and the tissue slices were incubated in the CO_2 incubator for several hours. The tissue slices were quickly washed with phosphate-free Tyrode buffer and frozen in liquid nitrogen. The tissue was thawed and homogenized at 4 °C with 1.0 mL of a solution containing 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethanesulfonyl fluoride, and 2% aprotinin. Insoluble materials were removed by centrifugation at 15000*g* for 20 min at 4 °C, and immunoprecipitation of filamin was performed with a polyclonal goat anti-chicken gizzard filamin antibody. Lysates (0.5 mL) were incubated first with 30 μL of the antibody for 90 min at 0 °C and then with 0.15 mL of a solution containing 50% (v/v) protein A-Sepharose, 20 mM Tris/HCl (pH 7.4), and 0.15 M NaCl for 15 min. Immune complexes were collected by centrifugation and washed four times with the washing buffer containing 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, and 50 mM NaF. Bound protein was released from the particles by boiling for 2 min in 60 μL of SDS sample buffer and analyzed by SDS−PAGE followed by autoradiography.

Phosphoamino Acid Analysis. The phosphoamino acid composition was analyzed as described previously (Ohta et al., 1989) with some modifications. Polyacrylamide fragments containing ^{32}P-labeled filamin were excised, washed extensively with 25% (v/v) isopropyl alcohol followed by 10% (v/v) methanol, and lyophilized. The dried gel slices were incubated at 37 °C for 24 h in 4 mL of 50 mM NH_4HCO_3 (pH 8.0) containing 100 μg/mL of trypsin and 50 μg/mL of chymotrypsin; the samples were centrifuged at 1000*g* for 5 min, and the supernatants were collected and lyophilized. The dried supernatant samples were dissolved in 200 μL of 6.0 N HCl and hydrolyzed for 1 h at 110 °C. After acid hydrolysis, the samples were dried and dissolved in 20 μL of a solution containing phosphoserine, phosphothreonine, and phosphotyrosine (1 mg/mL each) as markers. Phosphoamino acids were separated by high-voltage electrophoresis at 1.5 kV for 20 min on TLC plates (100 μm, 20 × 20 cm microcrystalline cellulose glass-backed plates without fluorescent indicators; E. M. Science, NJ) using pyridine/glacial acetic acid/H_2O (1:10:189, v/v). The positions of the phosphoamino acids were determined by ninhydrin staining and by autoradiography.

Measurements of F-Actin Gelation. Increasing concentrations of filamin, previously incubated with or without CaM kinase II for 60 min at 30 °C, were mixed with 12 or 23.8 μM actin in 100-μL capillary tubes (Clay Adams, NJ) at 25 °C in 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.43 mM EGTA, 0.2 mM $CaCl_2$, and 0.46 mM ATP. The time required for a 0.7 mm diameter stainless steel ball to fall 8.0 cm was measured after 60 min (Maclean-Fletcher et al., 1980; Hartwig et al., 1992). Filament cross-linking was also quantitated in a low-speed centrifugation assay (Brotschi et al., 1978) with some modifications. Untreated and phosphorylated filamins were incubated with 12 μM actin at 25 °C in Eppendorf test tubes (1.5 mL) or small cellulose propionate ultracentrifuge tubes (5 × 20 mm, Beckman) in a buffer composed of 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.43 mM EGTA, 0.2 mM $CaCl_2$, and 0.46 mM ATP. After 1 h, the samples were centrifuged at 10000*g* for 30 min at 25 °C. The supernatants were carefully removed from the actin pellets. Both supernatants and pellets were dissolved in SDS sample buffer to equal final volumes (100 μL), boiled for 3 min, and displayed by SDS−PAGE followed by Coomassie blue staining. The relative intensity of the Coomassie blue stained actin band was determined by counting pixels after digitization on an HP 256 gray scale scanner using the NIH Image 1.41 software program.

F-Actin Binding Assay. Filamin was incubated with CaM kinase II in the presence or absence of Ca^{2+} (1.0 mM Ca^{2+} or 0.5 mM EGTA) and 3 μM calmodulin at 30 °C for 60 min in 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM [γ-^{32}P]ATP. Increasing amounts of phosphofilamin or untreated filamin were mixed with 2.2 μM actin at 25 °C in 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 5 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.5 mM EGTA, and 0.25 mM ATP (the final volume of the reaction mixture was 100 μL). After 60 min, filamin bound to F-actin was collected by sedimenting the F-actin in a Beckman Airfuge at 100000*g* (30 psi) for 15 min at 25 °C. Supernatants were carefully removed from the pellets, and the pellets were solublized with 100 μL of 1% SDS solution with occasional vortexing for 30 min at 25 °C. Both supernatants and solublized pellet solutions were mixed with 33 μL of SDS sample buffer, boiled for 3 min, and analyzed by SDS−PAGE followed by autoradiography. The relative intensity

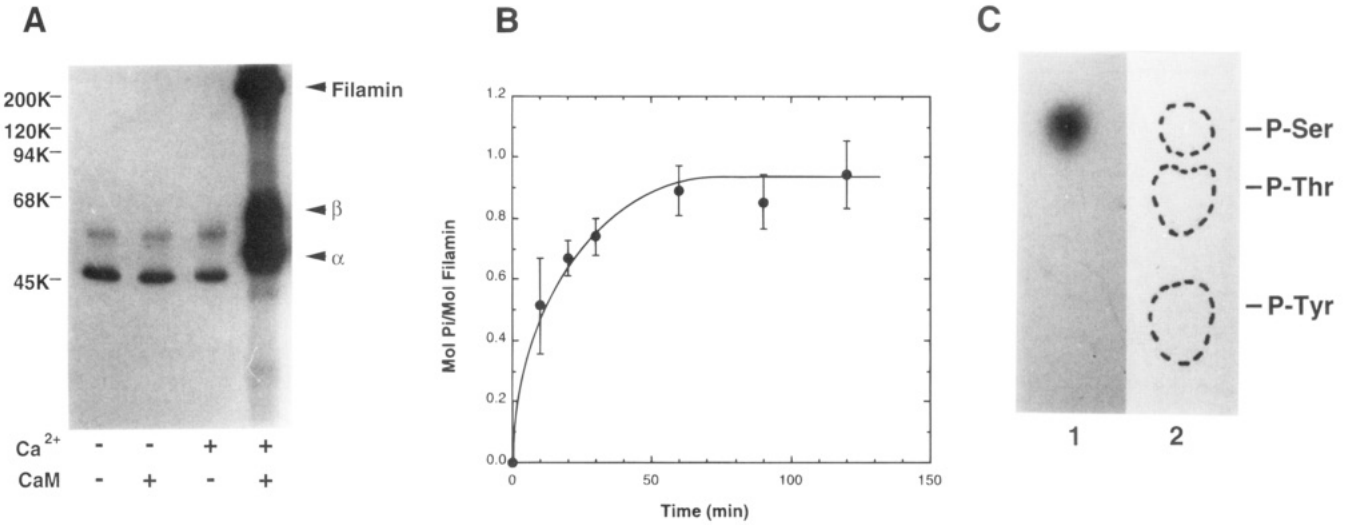

FIGURE 1: Phosphorylation of filamin by CaM kinase II. (A) Autoradiogram showing the covalent attachment of phosphate to filamin by CaM kinase II. Fifty microliters of chicken gizzard filamin (0.96 μM) was incubated with the rat brain CaM kinase II (0.027 μM) at 30 °C in 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, and 0.3 mM EGTA in the presence or absence of 1.0 mM $CaCl_2$ and 3.0 μM calmodulin. The reactions were terminated after 60 min by boiling the samples in SDS−PAGE sample buffer. The samples were analyzed by SDS−PAGE followed by autoradiography of the dried gel. Molecular weight markers include myosin (200 kDa), β-galactosidase (120 kDa), phosphorylase b (94 kDa), BSA (68 kDa), and ovalbumin (45 kDa). The positions of the phosphofilamin and the autophosphorylated subunits (α and β) of CaM kinase II are indicated. (B) Time course showing the incorporation of phosphate into filamin in the presence of CaM kinase II. Filamin (0.58 μM) was incubated with or without CaM kinase II (0.2 μM) as described in (A) except the concentration of ATP was 0.5 mM. Aliquots of each sample were taken at various time points and subjected to SDS−PAGE; the Coomassie-stained filamin bands were excised, and their ^{32}P content was determined in a scintillation counter. Each point of the graph shows the mean value ± SE for three separate measurements. (C) Phosphorylation of filamin at serine residues *in vitro* by CaM kinase II. Phosphoamino acid analysis was performed as described in Experimental Procedures. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

of the Coomassie blue stained filamin subunit band was determined as above.

Gel Filtration Column Chromatography. Filamin (0.43 μM), incubated with CaM kinase II (0.15 μM) and 3 μM calmodulin in 1.0 mM $CaCl_2$ or 0.3 mM EGTA at 30 °C for 60 min in 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM [γ-^{32}P]ATP was applied to a 1.5 × 32 cm Bio-Gel A15 200−400M column equilibrated with 10 mM imidazole, pH 7.5, 0.6 M KCl, 10 mM EGTA, 1.0 mM DTT, and 0.1 mM PMSF at 4 °C. Included fractions (0.5 mL/tube) were analyzed by SDS−PAGE and silver staining (GELCODE, Pierce) for the filamin polypeptide. The gel was then dried and subjected to autoradiography. The relative intensities of silver stained bands and phosphorylated bands (autoradiogram) were determined as described above. The gel filtration column was calibrated with the molecular size markers β-amylase (200 K) and thyroglobulin (670 K).

Phosphatase Treatment. Untreated filamin or filamin phosphorylated by CaM kinase II in 20 mM Tris/HCl (pH 7.4), 90 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.3 mM EGTA, 1.0 mM $CaCl_2$, and 3 μM calmodulin for 60 min at 30 °C, was incubated with and without the catalytic subunit of protein phosphatase 2A (30 μg/mL) for 60 min at 25 °C. Phosphatase-treated samples were then incubated with 12 μM actin at 25 °C in 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.43 mM EGTA, 0.2 mM $CaCl_2$, and 0.46 mM ATP. Actin cross-linking activity was quantitated after 60 min by centrifugation at 10000*g* for 30 min at 25 °C. The supernatants were carefully removed from the pellets, and the pellets were solublized with SDS stop solution to equal final volumes (100 μL), boiled for 3 min, and analyzed by SDS−PAGE followed by Coomassie blue staining.

Two-Dimensional Phosphopeptide Mapping. Polyacrylamide fragments containing ^{32}P-labeled filamin were excised and digested with trypsin (50 μg/mL) as described (Boyle et al., 1991). Phosphopeptides were spotted on TLC plates (20 × 20 cm microcrystalline cellulose adsorbent without fluorescent indicator, Kodak) and separated by electrophoresis at pH 1.9 (acetic acid/formic acid/water, 15:5:80 (v/v)) for 60 min at 1.5 kV and 4 °C. Chromatograms were dried and developed in a second dimension in 1-butanol/pyridine/acetic acid/water, 32.5:25:5:20 (v/v). Peptides were visualized by using a PhosphorImager (Molecular Dynamics) and the Image Quant (Molecular Dynamics).

Other Methods. SDS−PAGE was performed by the method of Laemmli on 5−15% gradient or 10% slab gels (Laemmli, 1970). Total protein was determined by the method of Bradford (Bradford, 1976) using BSA as the standard.

RESULTS

CaM Kinase II Phosphorylates Filamin in Vitro. Figure 1A shows that purified rat brain CaM kinase II phosphorylates chicken gizzard filamin in a Ca^{2+}- and calmodulin-dependent manner *in vitro*. Maximal incorporation of phosphate was determined to be 0.9 mol of phosphate per mole of filamin dimer (Figure 1B). No proteolysis of filamin occurred during treatment of filamin with CaM kinase II or in the subsequent incubations with actin as judged by the integrity of the filamin polypeptide after SDS−PAGE and Coomassie blue staining (data not shown and Figure 5A). Phosphoamino acid analysis of filamin phosphorylated by CaM kinase II revealed serine to be the major phosphorylated residue (Figure 1C).

The Actin Cross-Linking Activity of Phosphofilamin Is Decreased. Filamin, phosphorylated with CaM kinase II to a maximal phosphate stoichiometry of 1 mol of phosphate per mole of filamin dimer, has diminished actin filament

cross-linking activity. Filamin cross-links actin filaments into three-dimensional gel networks. The gel point of a solution of actin filaments is defined as the critical concentration of a cross-linking protein required to form a gel. This value can be easily determined using viscometry by adding increasing amounts of filamin to a constant actin filament concentration as the point where the viscosity abruptly increases. As shown in panels A and B of Figure 2, addition of filamin to actin molar ratios of ≥ 1 to 80 gels actin filament solutions. When filamin was incubated with CaM kinase II under phosphorylation conditions, higher filamin concentrations were required for gelation and only >1 to 60 molar ratios of phosphofilamin to actin gelled the actin solution (Figure 2B). CaM kinase II alone did not interfere with actin polymerization (data not shown). The reduction in actin filament cross-linking by phosphofilamin was confirmed by a sedimentation assay. Cross-linked actin filaments sediment more rapidly than free filaments at low G-forces (Brotschi et al., 1978; Rockwell et al., 1984). Phosphofilamin was less effective than unphosphorylated filamin in increasing the sedimentation rate of actin filaments, and substantial amounts of phosphofilamin remained in the supernatants after centrifugation at 10000*g* for 30 min (Figure 2C). Four times more phosphofilamin was required to sediment the equivalent amount of actin pelleted by untreated filamin (Figure 2D). These results demonstrate that phosphorylation by CaM kinase II decreases the potency of filamin in cross-linking actin filaments.

The reduction in filamin's actin filament cross-linking activity is due to its phosphorylation rather than a direct interaction with CaM kinase II. First, CaM kinase II treatment was effective only when Ca^{2+} and calmodulin were present in the phosphorylation reaction (Figure 3). Second, even in the presence of Ca^{2+} and calmodulin, ATP was necessary for CaM kinase II to inactivate filamin (Figure 3). Third, heat-inactivated CaM kinase II failed to phosphorylate filamin or diminish its cross-linking activity (Figure 3). Lastly, the inhibitory effect of CaM kinase II-mediated phosphorylation on actin filament cross-linking was reversed by treatment with phosphatase. As shown in Figure 4A, protein phosphatase 2A dephosphorylated filamin previously phosphorylated by CaM kinase II. Under the standard incubation conditions, protein phosphatase 2A removed ~70% of radioactive phosphate incorporated by CaM kinase II from filamin within 30 min (Figure 4A). To determine whether dephosphorylated filamin recovered its actin filament cross-linking activity, thereby confirming phosphate addition to regulate filamin's cross-linking activity, phosphofilamin was treated with protein phosphatase 2A. As shown in Figure 4B,C, treatment of phosphofilamin by protein phosphatase 2A restored the actin filament cross-linking activity of filamin to levels determined for the untreated protein (Figure 4B, lane 5, and Figure 4C, bar d). Protein phosphatase 2A alone did not alter filamin's actin cross-linking activity (Figure 4B, lane 4). These results demonstrate that the effect of CaM kinase II on filamin's function is mediated by a specific phosphorylation and that removal of this added phosphate reverses the inhibitory effect of CaM kinase II.

Phosphofilamin Binds Less Efficiently to Actin Filaments. To determine whether phosphorylation regulated filament cross-linking by changing the interaction of filamin with actin filaments, the kinetics for phosphofilamin binding to actin filaments were determined. Figure 5A shows that phosphorylation of filamin decreased its affinity for actin filaments. Actin filament bound filamin was separated from free filamin by sedimentation of actin filaments at 100000*g*. Phosphofilamin alone did not sediment at this G-force (Figure 5A, lanes 5 and 10). When filamin was phosphorylated by CaM kinase II prior to incubation with actin, the amount of the unbound filamin protein remaining in the 100000*g* supernatant increased compared to filamin incubated with CaM kinase II but in the absence of Ca^{2+} or calmodulin (Figure 5A, lanes 1−4). This decrease in the amount of filamin bound to actin occurred only when Ca^{2+}, calmodulin, and ATP were present in the phosphorylation reaction (data not shown). When filamin was incubated with a heat-inactivated CaM kinase II, filamin was not phosphorylated, and its sedimentation with actin filaments was indistinguishable from that of control (untreated) filamin (data not shown). The binding of both phosphofilamin and filamin to actin filaments saturated at molar ratios of filamin to actin of 1:22 (Figure 5B) (Hartwig & Stossel, 1981). Scatchard analysis of the binding data showed that the affinity (K_d) of phosphofilamin for actin filaments, relative to the unphosphorylated protein, diminished ~2-fold from 3.2 to 6.9 μM (Figure 5C). These data suggest that the decreased actin filament cross-linking activity of phosphofilamin results from less binding of phosphofilamin to actin relative to untreated filamin.

We also determined whether the reduction of the phosphofilamin's actin cross-linking activity resulted from changes in aggregation or dissociation of its dimeric subunits by comparing the migration of phosphofilamin and untreated filamin through sizing columns. The elution profile of phosphofilamin was identical to that of the untreated protein, with both proteins eluting near a globular protein standard of 670 kDa (Figure 6) (Hartwig & Stossel, 1981). The elution profile of phosphofilamin detected by autoradiography of filamin bands also comigrated with that of total filamin, as determined by protein staining (data not shown). Moreover, phosphofilamin was not eluted in void fractions, suggesting large aggregates were not formed (data not shown). This result was also confirmed by dynamic light-scattering analysis of filamin/CaM kinase II mixtures after the phosphorylation reaction (data not shown).

Comparison of Sites in Filamin Phosphorylated in Vitro by CaM Kinase II and in Intact Chicken Gizzard. Sites phosphorylated in chicken gizzard filamin by CaM kinase II *in vitro* were determined by two-dimensional tryptic phosphopeptide mapping. As shown in Figure 7A, tryptic digestion of filamin yielded three major phosphopeptides. To compare these phosphorylation sites to those in intact tissue, freshly isolated chicken gizzard tissue was incubated with [^{32}P]orthophosphate, and ^{32}P-labeled filamin was immunoprecipitated with anti-filamin IgG and analyzed by SDS−PAGE followed by autoradiography. Incorporation of phosphate into the filamin bands was detected after 2 h of incubation with [^{32}P]orthophosphate and was maximal after 4−6 h of incubation (data not shown). Therefore, ^{32}P-labeled filamin isolated from gizzard tissue labeled for 4 h was analyzed by two-dimensional phosphopeptide mapping. Tryptic digestion of phosphofilamin from gizzard tissue yielded several weak spots (Figure 7B), one of which is identical to spot 2 from filamin phosphorylated by CaM kinase II *in vitro* (Figure 7A).

DISCUSSION

The present study demonstrates filamin's actin filament cross-linking activity to be regulated through phosphorylation

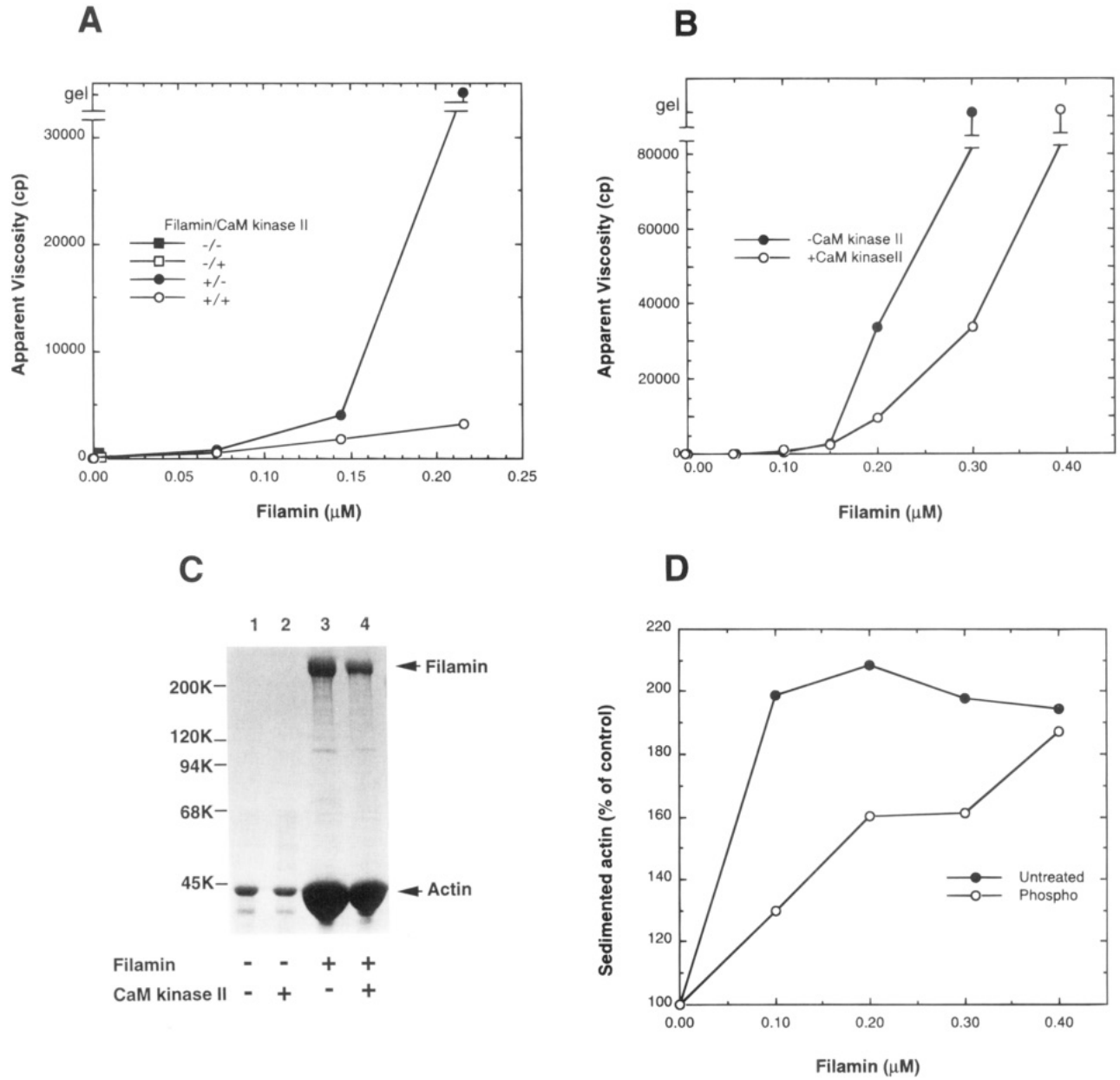

FIGURE 2: Effects of CaM kinase II-mediated phosphorylation on the actin filament cross-linking activity of filamin. (A) Determination of the critical gel point for phosphofilamin and untreated filamin. Filamin, phosphorylated by CaM kinase II as described in Figure 1B, was added to actin (12 μM) and incubated at 25 °C for 60 min in 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.43 mM EGTA, 0.2 mM $CaCl_2$, and 0.46 mM ATP. The graph shows actin alone (■), CaM kinase II and actin (□), filamin (untreated) and actin (●), and filamin treated with CaM kinase II (phosphofilamin) and actin (○). The low-shear falling ball assay was determined as described in Experimental Procedures. In this experiment, actin was purified by an additional polymerization and depolymerization cycle, and the critical filamin gelling concentration for 12 μM actin was 0.15 ~ 0.2 μM. No gelation was observed when filamin was phosphorylated with CaM kinase II at the tested filamin concentrations. (B) Determination of the critical gel point for phosphofilamin and untreated filamin. Phosphorylation of filamin was done as in (A). Phosphofilamin and untreated filamin were incubated with 23.8 μM actin for 60 min, and the low-shear falling ball assay was performed as in (A). The graph shows untreated filamin and actin (●) and phosphofilamin with actin (○). In this experiment, the critical gelling concentrations for 23.8 μM actin of untreated filamin and phosphofilamin were 0.2 ~ 0.3 μM and 0.3 ~ 0.4 μM, respectively. (C) Effect of the phosphorylation of filamin on actin filament cross-linking determined by low-speed sedimentation. Filamin (0.13 μM) was treated with (phosphofilamin, lane 4) or without (filamin, lane 3) CaM kinase II (0.04 μM) in phosphorylation conditions at 30 °C for 60 min. As controls, filamin or CaM kinase II was omitted from the phosphorylation reaction (lanes 1 and 2). The samples were mixed with actin (12 μM) and incubated as in (A) at 25 °C for 60 min. Cross-linked actin filaments (pellet) were collected by centrifugation at 10000g for 30 min at 25 °C and analyzed by SDS−PAGE and Coomassie blue staining. In this experiment, relative amounts of sedimented actin were as follows: lane 1, actin alone (100%); lane 2, actin plus CaM kinase II (83%); lane 3, actin plus untreated filamin (660%); lane 4, actin plus phosphofilamin (440%). In this experiment, actin was purified by an additional polymerization and depolymerization cycle. (D) Dose−response relationship of phosphofilamin or untreated filamin on actin filament cross-linking determined by low-speed sedimentation. Filamin was treated with (○) or without (●) CaM kinase II as in (C), mixed with actin (12 μM), and incubated at 25 °C for 60 min. Cross-linked actin filaments (pellet) were collected by centrifugation at 10000g for 30 min at 25 °C and analyzed by SDS−PAGE and Coomassie blue staining as in (C). In this experiment, actin was purified by an additional polymerization and depolymerization cycle, and polymerization of actin and centrifugation were performed using small centrifuge tubes as described in Experimental Procedures. Values are the mean of two experiments and are expressed as percentage of controls.

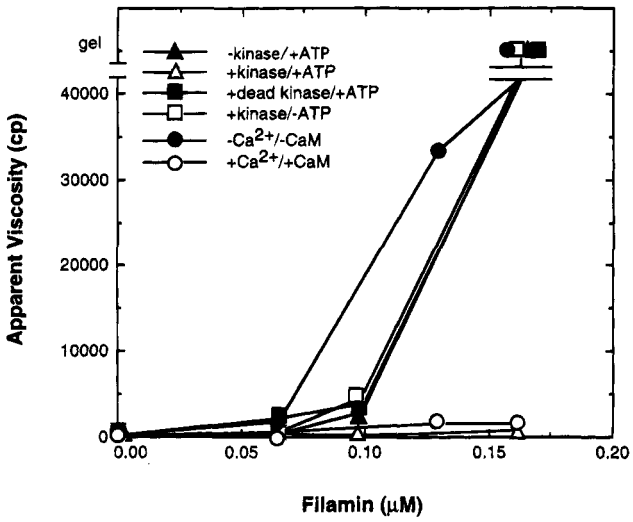

FIGURE 3: Effects of various conditions on the cross-linking activity of filamin. Filamin (1.0 μM) was incubated with CaM kinase II (0.15 μM) in the presence or absence of Ca^{2+} (1 mM $CaCl_2$ or 0.5 mM EGTA) and calmodulin (3 μM) under phosphorylation conditions for 60 min: (□) ATP (0.5 mM) was omitted from the reaction mixture; (■) CaM kinase II was inactivated by heating it to 100 °C for 5 min and then incubated with filamin in the presence of Ca^{2+} and calmodulin. Treated filamin was further incubated with actin (12 μM) at 25 °C for 60 min. Then, actin-cross-linking activity of filamin was measured by the low-shear falling ball assay as in Figure 2A. In this experiment, actin was purified by an additional polymerization and depolymerization cycle.

by CaM kinase II *in vitro*. CaM kinase II is a serine/threonine-specific protein kinase activated by Ca^{2+} and calmodulin that undergoes autophosphorylation in response to a variety of cellular agonists. It has been identified in organisms from yeast to mammals (Nairn et al., 1985; Edelman et al., 1987; Hanson & Schulman, 1992). In vertebrates, CaM kinase II is highly abundant in brain tissue, but many nonneuronal isozymes have been identified and/or purified from other tissues (Ohta et al., 1988). Although these isozymes differ in molecular weight and subunit composition, they have similar substrate specificity and recognize a R-X-X-S/T consensus phosphorylation sequence. Several lines of evidence suggest that CaM kinase II may be involved in the regulation of cellular cytoskeletal structure and function. Transfection of neuroblastoma cells with cDNA for CaM kinase II promotes spreading (Goshima et al., 1993). Chemotropic behavior of nerve growth cones may be modulated by CaM kinase II (Zheng et al., 1994). A large fraction of brain CaM kinase II partitions the detergent-insoluble cytoskeleton (Edelman et al., 1985; Schulman, 1988). Many cytoskeletal proteins are phosphorylated and thereby modulated by CaM kinase II *in vitro*. These include synapsin I (Greengard et al., 1993), MAPs (Yamamoto et al., 1983) and intermediate filament proteins (Tokui et al., 1990).

Although CaM kinase II has a relatively broad substrate specificity, our study demonstrates chicken gizzard filamin to be phosphorylated at specific sites by CaM kinase II. Three major phosphopeptides were found by two-dimensional phosphopeptide mapping of phosphofilamin, suggesting that one or more contain the phosphates responsible for the functional modification of filamin. We found filamin to be phosphorylated at multiple sites in tissue slices of chicken gizzard. One of these phosphopeptides colocalized with one of the major phosphopeptides generated *in vitro* by CaM kinase II (spot 2). Although the degree of phosphorylation of the peptide in resting tissue was low compared to that of other phosphopeptides *in vivo*, it is possible that stimulation of smooth muscle could increase the level of its phosphorylation. It is not clear at present whether this site is critical for the modulation of filamin function. Further analysis (i.e., sequencing and *in vitro* mutagenesis of phosphorylation sites) is necessary to determine the critical modification site. It is also unclear whether CaM kinase II is responsible for the phosphorylation of this site in intact gizzard. Nonetheless, our results show that the phosphorylation of specific polypeptides, which could modulate actin-binding activity of filamin, occurs in intact gizzard.

Phosphorylation by CaM kinase II diminishes filamin's actin filament cross-linking activity. One trivial explanation of this inactivation is that it is due to a formation of large aggregates of phosphofilamin or phosphofilamin−CaM kinase II. This is unlikely because (1) phosphofilamin did not sediment by itself or with autophosphorylated CaM kinase II, (2) phosphofilamin eluted with untreated filamin through a size-exclusion gel column, and (3) phosphofilamin−CaM kinase II aggregates were not detected by light scattering. The present study suggests that the change in actin cross-linking potency of filamin results from a reduction of filamin's affinity for actin filaments following phosphorylation. CaM kinase II treatment diminished the affinity of filamin for actin filaments by 2-fold. The decreased affinity of phosphofilamin could hypothetically result from altered (1) actin-binding properties through the attachment of phosphate within the amino-terminal actin-binding sites of filamin; (2) monomer/dimer equilibrium through phosphorylation within the carboxyl-terminal self-association site; or (3) molecular conformation changes induced by phosphorylation at sites along the backbone of the filamin molecule. Calpain cleavage experiments indicate that the carboxyl-terminal self-association site is not phosphorylated by CaM kinase II (data not shown). Calpain cleaves chicken gizzard filamin into a 10-kDa carboxyl-terminal subfragment and 240-kDa subfragments. The large subfragment retains actin filament binding activity, but cannot cross-link actin filaments because it is monomeric (Davies et al., 1978, 1980). The carboxyl-terminal subfragment, on the other hand, contains the self-association site of filamin and ABP-280 (Weihing, 1988; Gorlin et al., 1990; Hock et al., 1990). Since radiolabel remains with the large, not the small, subfragment after cleavage by calpain, phosphorylation of chicken gizzard filamin occurs outside of the self-association site. Chicken filamin isoforms expressed in Muller glial cells contain five predicted phosphorylation sites for CaM kinase II, all located outside of the self-association site (Barry et al., 1993). Whether phosphorylation occurs within the filamin's ~280-residue actin-binding domain or elsewhere on its elongated backbone remains to be determined.

Although filamin is believed to be a homodimer, the maximal stoichiometry for phosphate incorporation by CaM kinase II was 1 mol of phosphate per mole of filamin dimer. Since α-chymotryptic digestion of phosphorylated filamin generated several phosphopeptides, functional modification of filamin should be caused by the incorporation of less than 1 mol of phosphate per mole of filamin dimer. This suggests that once CaM kinase II phosphorylates the specific site in one filamin subunit, phosphorylation of the other subunit does not occur. This did not appear to be due to endogenous phosphorylation of the other site in the purified protein. In this case, phosphatase 2A treatment should enhance the cross-

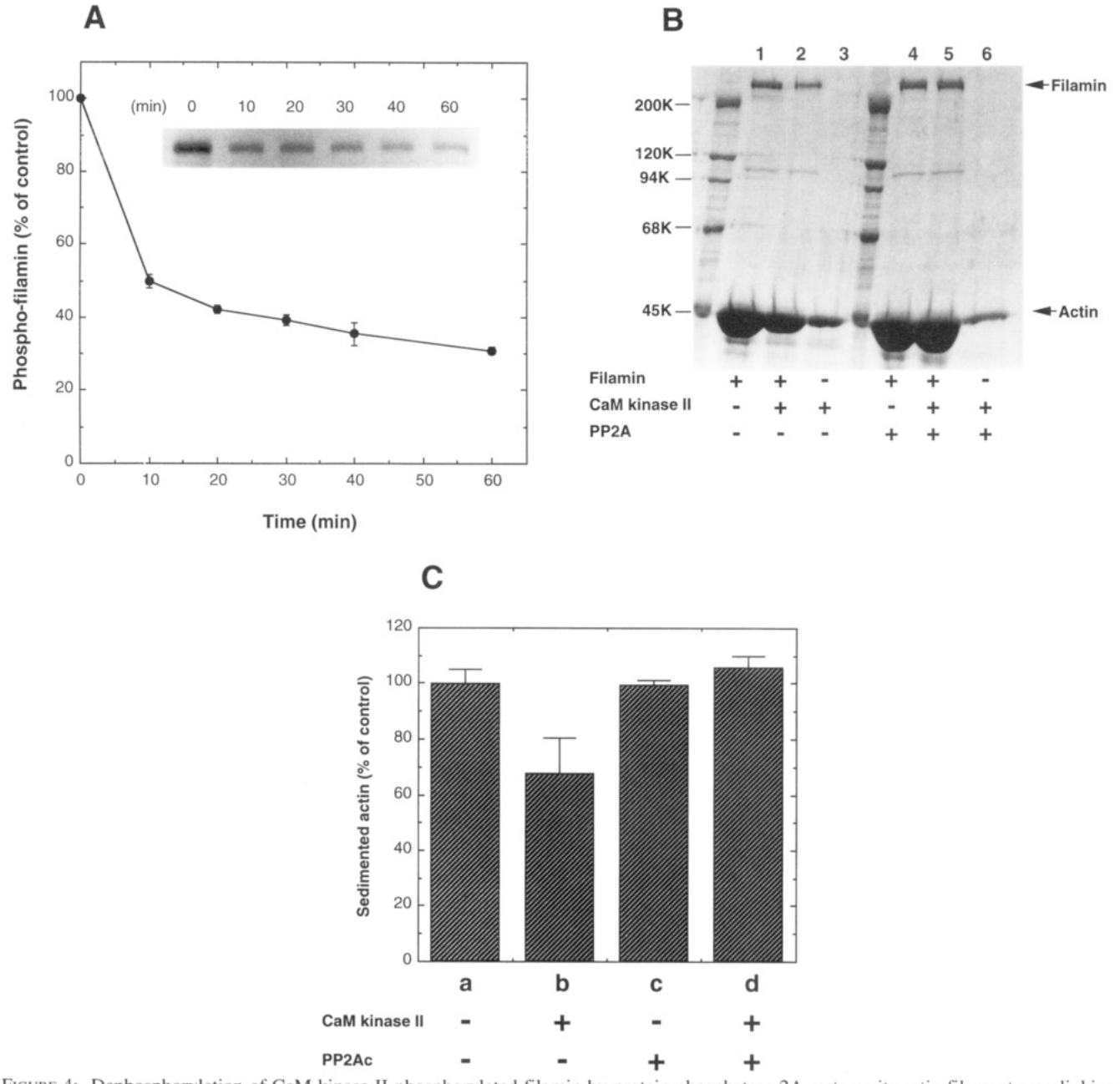

FIGURE 4: Dephosphorylation of CaM kinase II-phosphorylated filamin by protein phosphatase 2A restores its actin filament crosslinking activity to untreated levels. (A) Time course of the dephosphorylation of phosphofilamin by protein phosphatase 2A. Filamin, phosphorylated using CaM kinase II, was incubated with the catalytic subunit of protein phosphatase 2A (30 μg/mL) at 25 °C. At various times, aliquots (15 μL) were removed, 5 μl of SDS sample buffer was added, and the samples were boiled and subjected to SDS−PAGE followed by autoradiography. Each point of the graph shows the mean value ± SE for three separate experiments and expressed as percentage of control. The relative intensities of the filamin bands in the autoradiogram were determined. The inset shows a representative autoradiogram of filamin bands. (B) Restoration of phosphofilamin's actin filament cross-linking activity to basal levels by dephosphorylation. Filamin (0.13 μM), phosphorylated with CaM kinase II (0.04 μM) for 60 min at 30 °C (lanes 2, 3, 5, and 6) or untreated (lanes 1 and 4), was further incubated in the presence (lanes 4, 5, and 6) or absence (lanes 1, 2, and 3) of 30 μg/mL of the catalytic subunit of protein phosphatase 2A for 30 min at 25 °C. The filamin samples were then incubated with actin (12 μM) for 60 min at 25 °C and centrifuged at 10000g for 30 min, and the resulting pellets were displayed by SDS−PAGE. In lanes 3 and 6, no filamin was added. The relative quantities of sedimented actin are as follows: lane 1, 100%; lane 2, 58%; lane 3, 24%; lane 4, 104%; lane 5, 113%; and lane 6 21%. (C) Actin cross-linking activity of phosphofilamin or dephosphorylated filamin by low-speed sedimentation. The bars represent the relative quantities of actin sedimented by filamin at low G-force. Values are means ± SE (n = 3) and are expressed as percentage of control. In (B) and (C), actin was purified by an additional polymerization and depolymerization cycle.

linking activity of purified protein. Phosphorylation and inactivation of the actin-binding site on one subunit, but not the other, would be sufficient to inhibit filament cross-linking. This would be expected to alter the amount of filamin bound to actin but would not provide a mechanism to sterically block CaM kinase II's access to the other actin-binding site, particularly since the actin-binding sites on filamin molecules are widely spaced (Tyler et al., 1980). Alternatively, phosphorylation of filamin's backbone near its self-association site could close the molecule by promoting lengthwise subunit aggregation. A resulting conformational rearrangement of the actin-binding sites could alter their affinity for actin and block CaM kinase II access to the consensus site on the opposing chain. It is also possible that purified filamin molecules are mixtures of isoforms and that only some of them contain phosphorylation sites for CaM kinase II and

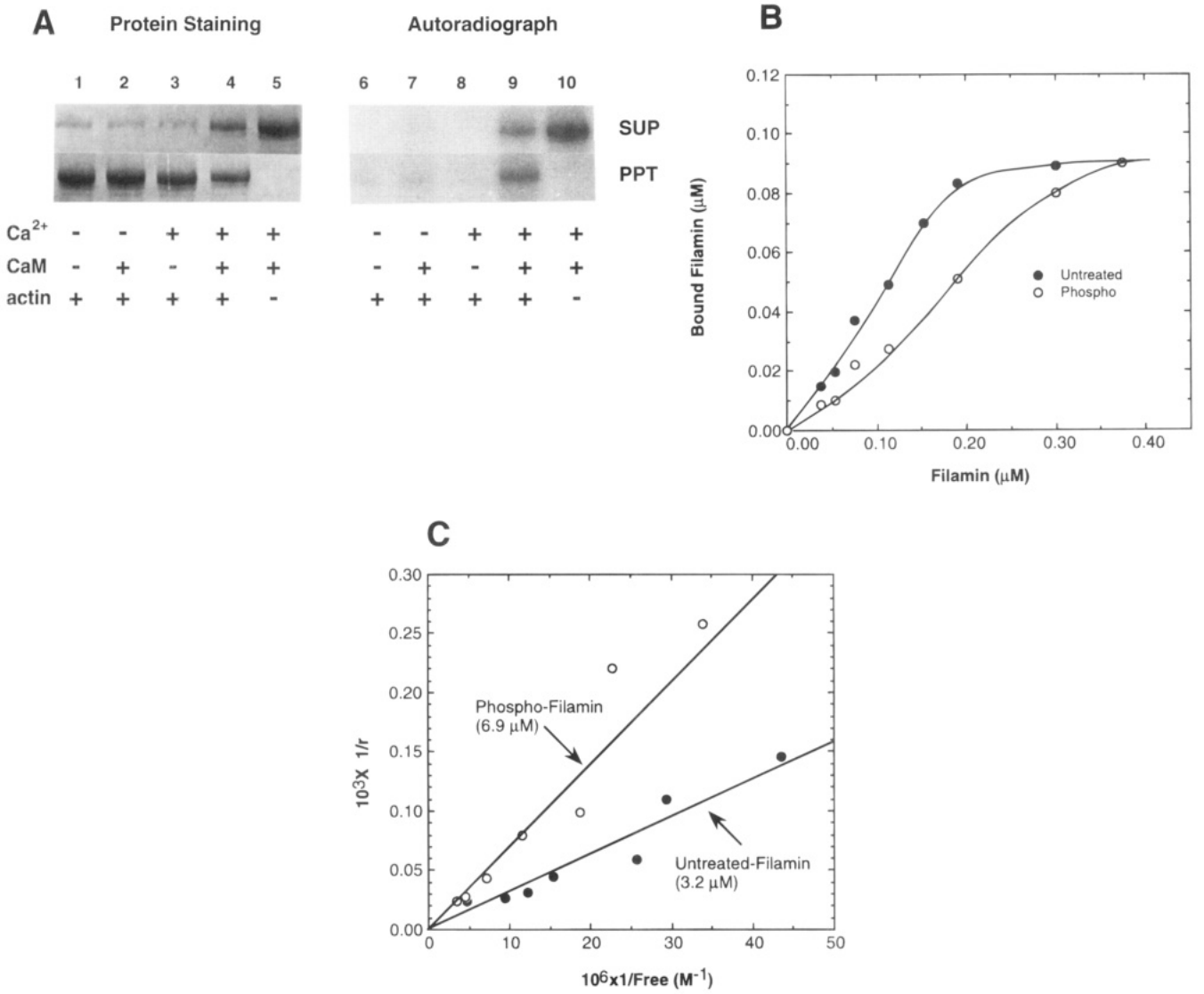

FIGURE 5: Reduction of actin filament binding after phosphorylation of filamin by CaM kinase II. (A) Filamin was phosphorylated by CaM kinase II in the presence or absence of Ca^{2+} (1.0 mM $CaCl_2$ or 0.5 mM EGTA) and calmodulin (3 μM) (lanes 1−4 and 6−9) under phosphorylation condition with [γ-^{32}P]ATP for 60 min. After the phosphorylation reaction was complete, G-actin was added to a final concentration of 2.2 μM, and the mixtures were incubated at 25 °C for 60 min and then centrifuged at 100000*g* for 15 min. As a control, phosphorylated filamin was centrifuged without actin (lanes 5 and 10). The supernatants were carefully removed from the tubes, and the supernatants (SUP) and pellets (PPT) were dissolved in an equivalent final volume of SDS−PAGE sample buffer and electrophoresed on 5−15% SDS−PAGE gels, which was followed by autoradiography of the dried gels. Filamin bands on the Coomassie blue stained gel (lanes 1−5) and the corresponding autoradiogram (lanes 6−10) are shown. Abbreviations: SUP, supernatants; PPT, pellets; Protein Staining, Coomassie blue staining; Autoradiograph; autoradiogram of Coomassie blue stained gel. (B) Dose dependence of the binding of phosphofilamin and untreated filamin to actin filaments. Filamin (0.75 μM) was phosphorylated using CaM kinase II (0.25 μM) in the presence (○) or absence (●) of Ca^{2+} and calmodulin. Increasing concentrations of filamin were incubated with G-actin (2.2 μM) in 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM EGTA, 5 mM $MgCl_2$, and 0.25 mM ATP for 60 min at 25 °C. The filamin−actin samples were centrifuged at 100000*g* for 15 min. The resultant pellets and supernatants were separated and analyzed by SDS−PAGE. The bound (pelleted) and unbound (soluble) filamin concentrations were determined from the pixel density of the stained gel bands after digitization using an HP scanner and the NIH Image Analyzer software. (C) Scatchard plot analysis of the specific binding of phosphofilamin and untreated filamin to actin filaments. The data was from (B). *r* is the bound filamin per total actin (2.2 μM).

can be regulated. Determination of the critical phosphorylation site(s) in filamin should help to unveil the mechanism of regulation.

Our present study demonstrated that the treatment of phosphofilamin with protein phosphatase 2A restored its actin filament cross-linking activity to the untreated levels, confirming regulation by phosphorylation−dephosphorylation. Phosphatase 2A treatment alone did not alter filamin's actin cross-linking activity. Zhuang et al., however, reported that *E. coli* phosphatase or endogenous platelet phosphatase treatment diminished the actin filament cross-linking activity of human platelet ABP-280 (Zhuang et al., 1984) and, conversely, that phosphorylation increased its activity; however they did not show phospho-ABP, after treatment with protein phosphatases, to regain its actin cross-linking activity. Whether this reflects different phosphorylation sites or differences between smooth muscle filamin and platelet ABP remains to be determined. The presence of filamin isoforms in avian cells and tissues has been described (Pavalko et al., 1989; Gomer & Lazarides, 1983a,b). ABP-280 has at least three isoforms composed of similar but distinct primary structures (Maestrini et al., 1993). Whether these isoforms can be differentially regulated by CaM kinase II remains to be determined. It is also possible that multiple protein kinases phosphorylate ABP-280 or filamin and induce the opposite effect on their actin filament cross-linking

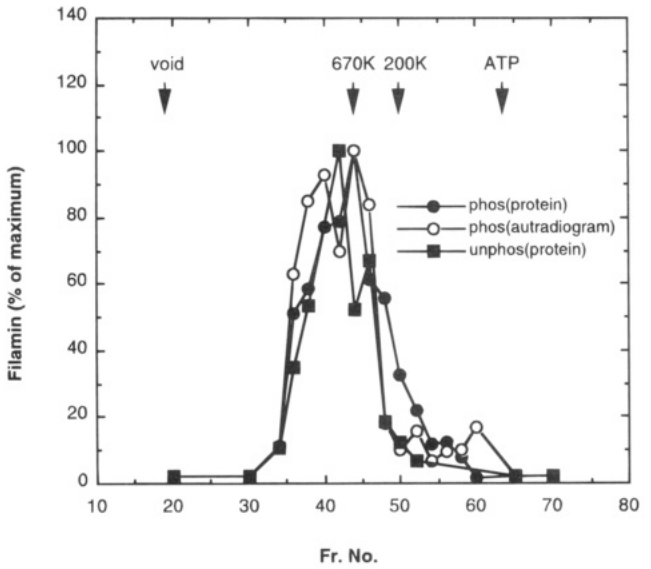

FIGURE 6: Molecular sizing of unphosphorylated and phosphorylated filamin. Filamin was phosphorylated by CaM kinase II in the presence or absence of Ca^{2+} and calmodulin. The samples were applied at 4 °C to a Bio-Gel A15M column. Eluted fractions were analyzed by SDS−PAGE and silver staining. The gel was dried and subjected to autoradiography. The relative intensities of stained bands (● and ■) and phosphorylated bands (○) of filamin were determined and are shown as the percentage of maximal intensity as described under Experimental Procedures. The elution points of β-amylase (200 kDa), tyroglobulin (670 kDa), and ATP are indicated.

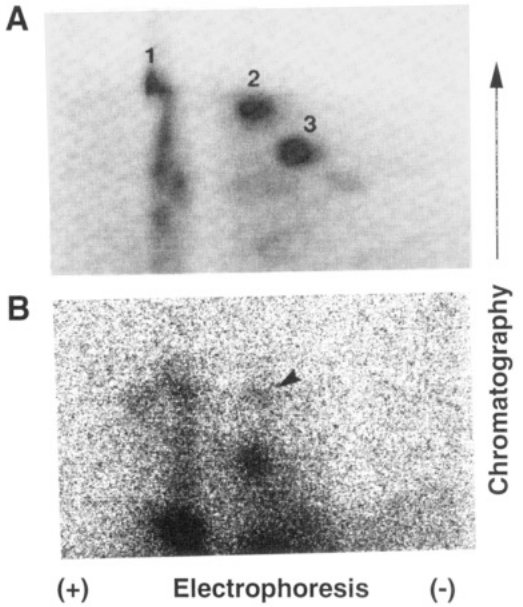

FIGURE 7: Two-dimensional tryptic phosphopeptide analysis of filamin. Purified chicken gizzard filamin was phosphorylated by CaM kinase II, or filamin was immunoprecipitated from ^{32}P-labeled checken gizzard. Phosphorylated filamins were separated by SDS−polyacrylamide gel electrophoresis and isolated as described under Experimental Procedures. After tryptic digestion, phosphopeptides were separated in two dimension on cellulose plates and detected by using a PhosphorImager as described under Experimental Procedures. (A) Tryptic peptide map of chicken gizzard filamin phosphorylated *in vitro* by CaM kinase II. (B) Tryptic peptide map of filamin from chicken gizzard. In (A) *in vitro* phosphorylated peptides are numbered. In (B) the spot which comigrates with spot 2 in (A) is indicated (arrowhead). The orientation of electrophoresis (+, −) and the direction of chromatography are shown.

activity. ABP-280 contains phosphorylation consensus sequences for various serine/threonine protein kinases other than CaM kinase II (Gorlin et al., 1990).

Sol−gel transitions of cortical actin filaments are believed to be critical for rapid cell movements (Stossel, 1993). The primary function of filamin in cells is to cross-link actin filaments into a gel network. Regulation of the sol−gel transition can be mediated by shifting the critical gelling concentration of a cross-linker. Below the critical gelling concentration, actin filaments are in a sol state, while above the critical gelling concentration, gelation of actin filaments occurs abruptly. Therefore, a subtle change of the critical gelling concentration of filamin could result in a large change in the mechanical property of the actin network. Although CaM kinase II causes only a 2-fold change in filamin's K_d to actin filaments, it increased the critical gelling concentration of filamin and resulted in a large difference of the network properties between unphosphorylated and phosphofilamin *in vitro*. Since the concentration of filamin in smooth muscle is about 6 μM (Wang, 1977), a shift of K_d from 3 to 6 μM would affect the amount of filamin bound to actin *in vivo* and, therefore, the mechanical properties of the actin cytoskeleton. It should be mentioned that the concentration of actin and the length distribution of actin filaments also dramatically affect the sol−gel transformation, and actin-fragmenting proteins, as well as actin-depolymerizing proteins, can control the sol−gel transition of actin networks in cells (Stossel, 1989). Nonetheless, our present study suggests that the modulation of cross-linking of filamin by phosphorylation can occur in cells and opens a new regulatory cascade to control the mechanical properties of cytoplasmic actin networks.

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