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Rapid Communication

B-50/GAP-43 Binds to Actin Filaments Without Affecting Actin Polymerization and Filament Organization

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Abstract: To investigate a possible function of the nervous tissue-specific protein kinase C substrate B-50/GAP-43 in regulation of the dynamics of the submembranous cytoskeleton, we studied the interaction between purified B-50 and actin. Both the phosphorylated and dephosphorylated forms of B-50 cosedimented with filamentous actin (F-actin) in a Ca^{2+}-independent manner. Neither B-50 nor phospho-B-50 had any effect on the kinetics of actin polymerization and on the critical concentration at steady state, as measured using pyrenylated actin. Light scattering of F-actin samples was not increased in the presence of B-50, suggesting that B-50 does not bundle actin filaments. The number of actin filaments, determined by [^{3}H]cytochalasin B binding, was not affected by either phospho- or dephospho-B-50, indicating that B-50 has neither a severing nor a capping effect. These observations were confirmed by electron microscopic evaluation of negatively stained F-actin samples, which did not reveal any structural changes in the actin meshwork on addition of B-50. We conclude that B-50 is an actin-binding protein that does not directly affect actin dynamics. **Key Words:** B-50/GAP-43—Actin—Cytoskeleton—Actin-binding proteins—Protein kinase C.
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Growth cone motility and neurotransmitter release are closely correlated with dynamic rearrangements in the submembranous actin skeleton (Smith, 1988; Bernstein and Bamburg, 1989; Letourneau and Cypher, 1991). Both processes are profoundly affected by stimulation of protein kinase C (PKC) (Burgoyne et al., 1989; Bamburg and Bernstein, 1991; Luna and Hitt, 1992), but the mechanism by which PKC affects actin dynamics in growth cones and nerve endings is still largely unknown.

The only protein implicated so far in mediating the effect of PKC is the myristoylated alanine-rich C kinase substrate (MARCKS), an ubiquitous protein that binds calmodulin (CaM) and is phosphorylated during macrophage and neutrophil activation and neurosecretion (reviewed by Aderem, 1992; Blackshear, 1993). MARCKS is suggested to act as a reversible cross-bridge between actin and the plasma membrane and regulates the cross-linking of filamentous actin (F-actin) (Hartwig et al., 1992).

A nervous tissue-specific candidate for mediating the effects of PKC on actin dynamics is the growth-associated protein B-50 [also known as GAP-43, neuromodulin, and F1 (reviewed by Skene, 1989; Liu and Storm, 1990; Gispen et al., 1991; Strittmatter et al., 1992)]. This protein is highly expressed in growth cones and presynaptic terminals and colocalizes to a large extent with F-actin (H. B. Nielander et al., manuscript submitted for publication). B-50 is a good substrate for PKC and is able to bind CaM at low [Ca^{2+}] ($\leq 10^{-7}$ *M*). PKC-mediated phosphorylation and CaM binding, which occur in the same B-50 domain, are mutually exclusive. These properties have been implicated in nerve growth cone targeting and attachment to the substratum (Dent and Meiri, 1992; Widmer and Caroni, 1993), as well as in Ca^{2+}-induced neurotransmitter release from nerve terminals (Dekker et al., 1989). A conserved sequence at the COOH-terminus of B-50 has been suggested to interact with cytoskeletal elements (LaBate and Skene, 1989). In isolated growth cones B-50 immunoreactivity was detected in the actin-enriched membrane skeleton tightly bound to the extracellular substrate (Meiri and Gordon-Weeks, 1990). In chicken, B-50 was also demonstrated to be tightly bound to the actin-rich neuronal membrane skeleton (Moss et al., 1990).

Here we investigate whether B-50 binds to F-actin and affects actin dynamics, using methodologies previously used to investigate the interactions between actin and MARCKS (Hartwig et al., 1992) or synapsin I (Bähler and Greengard, 1987; Benfenati et al., 1992; Valtorta et al., 1992). We report

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Abbreviations used: CaM, calmodulin; Cyt-B, cytochalasin B; F-actin, filamentous actin; G-actin, globular (monomeric) actin; MARCKS, myristoylated alanine-rich C kinase substrate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; SDS, sodium dodecyl sulfate.

that B-50 is an actin-binding protein that does not directly affect actin polymerization and organization and that its binding is not directly modulated by either Ca^{2+} or PKC-mediated phosphorylation.

MATERIALS AND METHODS

Materials

N-(1-Pyrenyl)iodoacetamide was obtained from Molecular Probes (Eugene, OR, U.S.A.), [^{3}H]cytochalasin B ([^{3}H]Cyt-B; specific activity, 22 Ci/mmol) from Amersham (Buckinghamshire, U.K.), and ATP and CaM from Boehringer (Mannheim, F.R.G.). All other chemicals were from standard commercial suppliers. B-50 was purified from bovine brain according to the method of Schotman et al. (1989). Actin was purified from rabbit skeletal muscle and chemically modified with *N*-(1-pyrenyl)iodoacetamide as reported by Valtorta et al. (1992).

B-50 phosphorylation

B-50 (37.5 μg of protein in a final volume of 300 μl) was stoichiometrically phosphorylated with PKC (0.16 μg of protein) for 40 min at 30°C in a buffer containing 10 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 0.2 m*M* $CaCl_2$, 100 μ*M* ATP, and 20 μg/ml of phosphatidylserine, pH 7.4. B-50 was mock-phosphorylated using heat-inactivated (20 min, 100°C) PKC. The reaction was stopped by freezing (−20°C). Phosphorylation stoichiometry was checked by back-phosphorylation of heat-inactivated B-50 with PKC.

Cosedimentation assay

Actin (5 μ*M*) was polymerized in G-buffer (0.2 m*M* $CaCl_2$, 0.2 m*M* ATP, 0.5 m*M* NaN_3, 0.5 m*M* 2-mercaptoethanol, and 2 m*M* Tris-HCl, pH 8.0) with 30 m*M* KCl and 1 m*M* $MgCl_2$ for 3 h on ice before it was incubated with various concentrations of B-50 for 30 min at 20°C. In some experiments B-50 was added before polymerization. After 30 min of centrifugation in a Beckman TLA-100 rotor (100,000 rpm) at 20°C, the F-actin-containing pellet and globular (monomeric) actin (G-actin)-containing supernatant were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). Actin and B-50 were detected by protein staining with Coomassie Brilliant Blue and/or by immunoblotting (Van Hooff et al., 1988).

Fluorescence measurement of actin polymerization

Samples containing G-actin (5 μ*M*, 5% pyrenylated) were equilibrated in 700 μl of G-buffer for 10 min at 25°C before polymerization was triggered by addition of 30 m*M* KCl and 1 m*M* $MgCl_2$ (t = 0). In most experiments B-50 (0.25–1.0 μ*M*) and polymerization salts were added simultaneously. In some cases B-50 was preincubated with G-actin for 30 min before addition of the polymerization salts. Fluorescence was recorded using an LS50 spectrofluorometer (Perkin-Elmer, U.K.) with excitation and emission wavelengths at 365 and 407 nm (slits set at 2.5 and 10 nm), respectively (Valtorta et al., 1992).

Light scattering assay

Actin (3 μ*M*) was polymerized in the absence of B-50 for 2 h as described for cosedimentation assays. Light scattering intensity of F-actin samples before and after addition of B-50 was recorded at an angle of 90° using an LS50 spectrofluorometer with both excitation and emission wavelengths at 400 nm (slits set at 5 nm) and a 2% attenuator (Ando and Scales, 1985).

[^{3}H]Cyt-B binding

Actin (10 μ*M*) was polymerized in G-buffer with 30 m*M* KCl and 2 m*M* $MgCl_2$ in the absence or presence of B-50 (0.5 μ*M*) for 30 min at 20°C before adding [^{3}H]Cyt-B (30 n*M*). The samples were incubated for another 30 min at 20°C, and F-actin was precipitated as described for cosedimentation assays. Nonspecific [^{3}H]Cyt-B binding was defined as the binding in the presence of 10 μ*M* unlabeled Cyt-B (Benfenati et al., 1992).

Electron microscopy

Actin (5 μ*M*) was polymerized in G-buffer with 30 m*M* KCl and 1 m*M* $MgCl_2$ in the absence or presence of B-50 (0.5 μ*M*) for 30 min at 20°C. Samples (20 μl) were applied to Formvar-coated copper grids. After 40 s, the grids were rinsed with 10 drops of G-buffer and stained with 1% uranyl acetate for 30 s. Excess stain was drawn off with filter paper. Grids were dried before observing them in a Hitachi model H-7000 electron microscope (Valtorta et al., 1992).

RESULTS AND DISCUSSION

Several reports have suggested the existence of an interaction between B-50 and the actin-enriched membrane skeleton (LaBate and Skene, 1989; Meiri and Gordon-Weeks, 1990; Moss et al., 1990; Strittmatter et al., 1992). To characterize such an interaction, we studied the binding of B-50 to F-actin using a high-speed cosedimentation assay (Bähler and Greengard, 1987; Valtorta et al., 1992). After a 30-min incubation, polymerized actin was pelleted, whereas monomeric actin (G-actin) remained in the supernatant. B-50 cosedimented with preformed F-actin in a concentration-dependent manner, whereas it stayed in the supernatant when actin was omitted (Fig. 1). Identical results were obtained when G-actin was polymerized in the presence of B-50. The binding of B-50 to F-actin did not reach saturation at 1 μ*M* B-50 and 5 μ*M* actin and was not affected by omitting Ca^{2+} from the polymerization medium. The latter finding is in line with the observation that the association of B-50 with the actin-enriched membrane skeleton is not affected by the presence of excess EGTA (Meiri and Gordon-Weeks, 1990). PKC and mock-phosphorylated B-50 cosedimented in a similar manner, indicating that B-50 binds to actin filaments in a phosphorylation-independent fashion. Because of electrostatic repulsion—B-50 and actin are both acidic proteins and negatively charged at pH values of >6—it is unlikely that B-50 binds to F-actin in a nonspecific manner.

We then studied the effect of B-50 on the kinetics of actin polymerization using pyrenylated actin. The polymerization of actin (5 μ*M*) induced by 30 m*M* KCl and 1 m*M* $MgCl_2$ was evaluated by following the increase in fluorescence as a function of time. The presence of B-50 (0.5 μ*M*) did not change the duration of the nucleation phase and the rate of actin polymerization either in the presence or in the absence of Ca^{2+} (Fig. 2). In line with the findings of the cosedimentation assay, B-50 did not significantly affect the ratio of G- to F-actin at steady state, as evaluated from the plateau levels of fluorescence. Similar results were obtained when PKC-phosphorylated B-50 was used. Increasing the ionic strength (100 m*M* KCl) or adding CaM under conditions known to promote CaM binding to B-50 [0.25 μ*M* CaM in the presence of 2 m*M* EGTA (De Graan et al.,

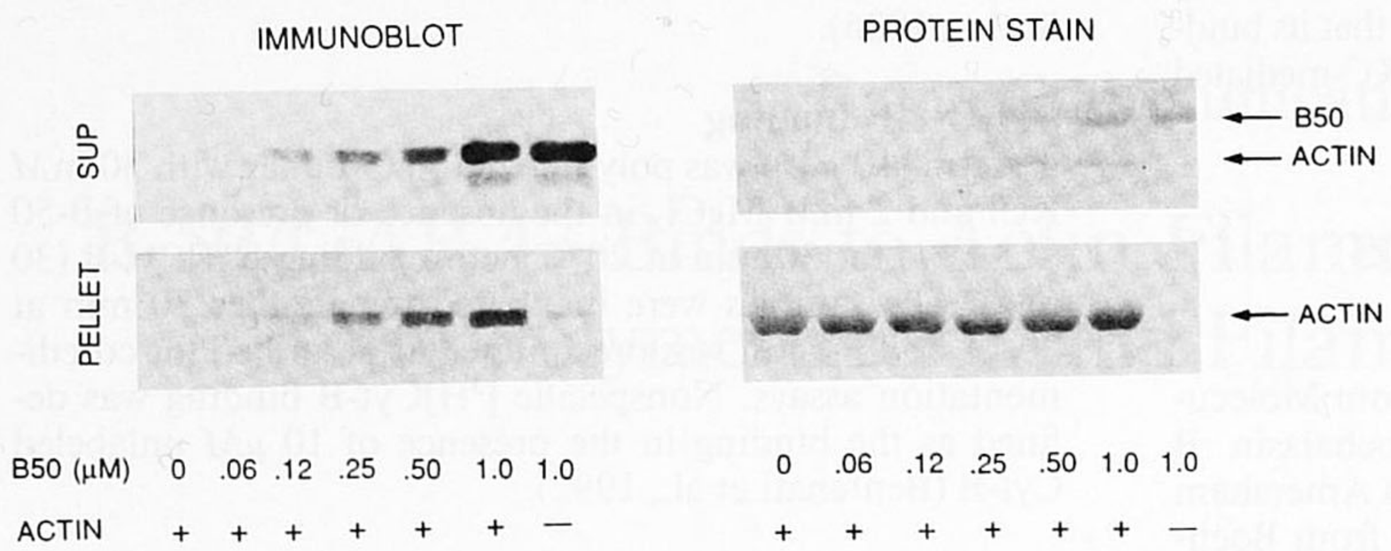


FIG. 1. Cosedimentation of B-50 with preformed F-actin. Actin (5 μM) was polymerized in G-buffer with 30 mM KCl and 1 mM $MgCl_2$ for 3 h on ice, before addition of the indicated amounts of B-50. After a 30-min incubation at 20°C, F-actin was pelleted by high-speed centrifugation. B-50 and actin in each supernatant and pellet were detected by B-50 immunostaining of western blots and by Coomassie Brilliant Blue staining after SDS-PAGE (10% gel). For immunoblotting, 33% of each supernatant and 75% of each pellet were loaded on the gel. The remainder of each sample was used for protein staining. Data shown are representative of three independent experiments.

1990)] also did not affect the kinetics of actin polymerization. Preincubation of G-actin with B-50 did not induce any significant polymerization and did not change the ability of actin monomers to nucleate after addition of KCl and $MgCl_2$ (Fig. 2, inset).

The effects of B-50 on the organization of the F-actin meshwork were studied using light scattering, [^{3}H]Cyt-B binding, and electron microscopy. Addition of 0.5 μM B-50 to polymerized actin did not increase the intensity of light scattering (data not shown), indicating that B-50 does not bundle actin filaments. The specific [^{3}H]Cyt-B binding to barbed ends of actin filaments is a measure of the number of actin filaments (Benfenati et al., 1992). Neither PKC nor mock-phosphorylated B-50 affected the specific [^{3}H]Cyt-B binding to preformed actin filaments, compared with the controls without B-50 (Fig. 3). This indicates that B-50 does not interfere with actin filament assembly, excluding the presence of a capping or severing effect. Electron microscopy of negatively stained F-actin samples confirmed the biochemical data. Indeed, the presence of either PKC or mock-phosphorylated B-50 did not induce any detectable difference in the structural organization of the F-actin meshwork (Fig. 4).

In summary, we have demonstrated that B-50 is an actin-binding protein in vitro. However, no effects of B-50 on actin polymerization and F-actin organization were observed. The inability of B-50 to bundle actin filaments suggests that B-50 does not possess multiple F-actin-binding sites. The interaction between B-50 and actin filaments may be localized at the COOH-terminal domain of the B-50 protein (LaBate and Skene, 1989). Mutated B-50 and site-specific B-50 antibodies that interfere with B-50 binding to F-actin might be helpful to confirm this possibility.

It remains to be established whether in growth cones and mature nerve terminals membrane-bound B-50 serves as an anchor protein for actin filaments. It is also possible that B-50 in vivo affects actin dynamics indirectly by regulating the activities of actin-binding proteins sensitive to phosphatidylinositol 4,5-bisphosphate, such as gelsolin and profilin (Bernstein and Bamburg, 1989; Forscher, 1989), or to CaM, such as caldesmon and fodrin (Bamburg and Bernstein, 1991). In fact, it has been reported previously that B-50 regulates phosphatidylinositol 4,5-bisphosphate turnover in a phosphorylation-dependent manner (Van Hooff et al., 1988; Gispen et al., 1991) or may serve as a local CaM store within growth cones (Liu and Storm, 1990) and nerve terminals (De Graan et al., 1990).

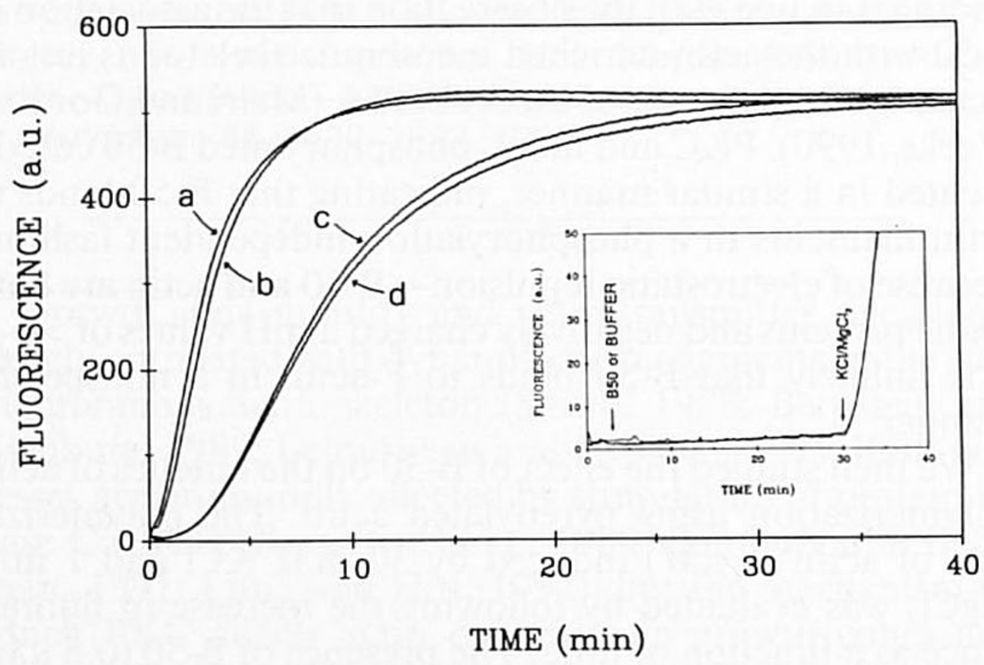


FIG. 2. Effect of B-50 on the kinetics of actin polymerization. The polymerization of G-actin (5 μM; 5% pyrenylated) was analyzed in the absence (curves b and c) or presence (curves a and d) of 0.5 μM B-50. Polymerization was triggered at time 0 by addition of 30 mM KCl and 1 mM $MgCl_2$ together with B-50 or buffer in the presence of 0.2 mM $CaCl_2$ (curves c and d) or in the presence of 2 mM EGTA (curves a and b; free [Ca^{2+}] of $<10^{-8}$ M). The increase in fluorescence is expressed in arbitrary units (a.u.). **Inset:** Effect of preincubation of B-50 with G-actin before triggering actin polymerization at 30 min. Experimental conditions were similar to those described for curves c and d.

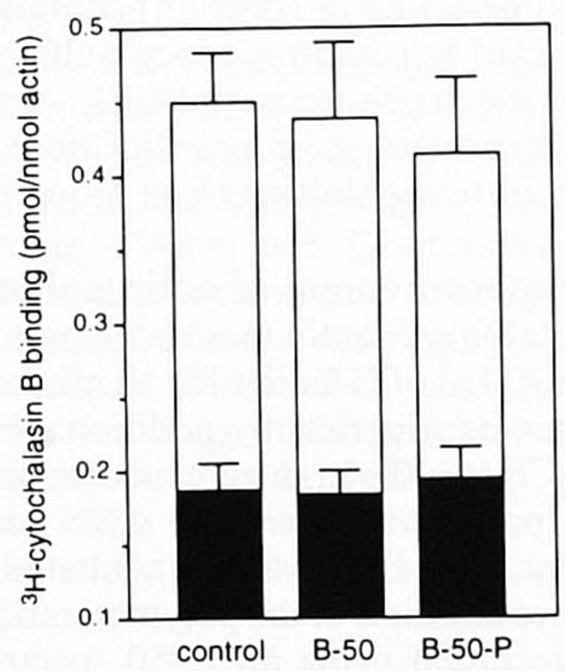
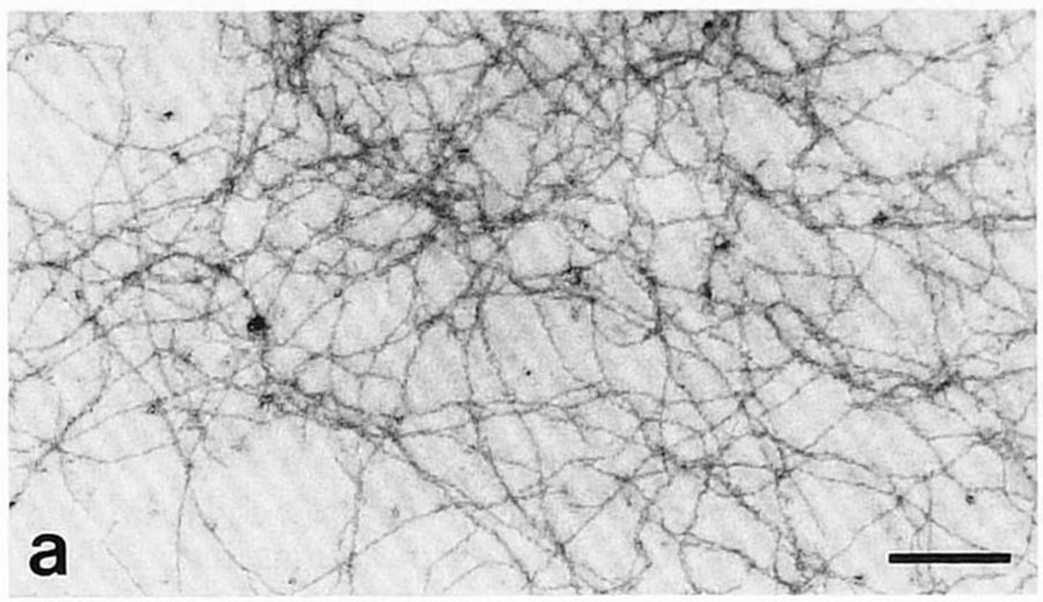
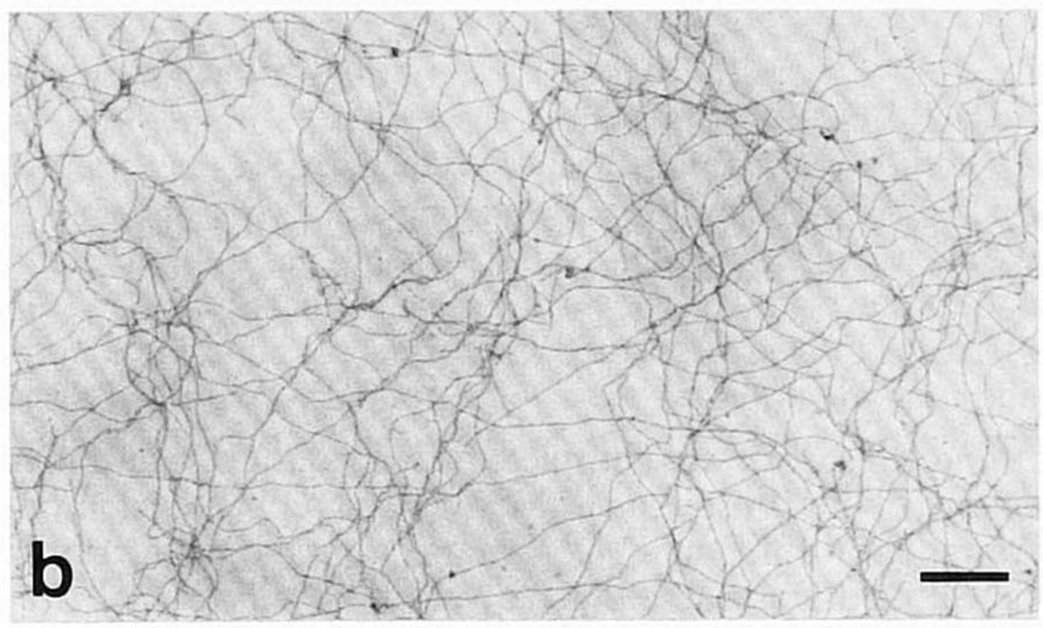


FIG. 3. Effect of B-50 on [^{3}H]Cyt-B binding to barbed ends of actin filaments. Actin (5 μM) was polymerized with 30 mM KCl and 2 mM $MgCl_2$ in the presence of buffer (control), mock-phosphorylated B-50 (0.5 μM; B-50), or phosphorylated B-50 (0.5 μM; B-50-P) for 30 min at 20°C before addition of [^{3}H]Cyt-B (30 nM). After an additional 30-min incubation, [^{3}H]Cyt-B bound to F-actin was quantified by high-speed centrifugation (open columns). Nonspecific [^{3}H]Cyt-B binding was determined as the binding in the presence of 10 μM unlabeled Cyt-B (solid columns). [^{3}H]Cyt-B binding, expressed as picomoles per nanomole of actin, are mean ± SEM (bars) values of four replications.





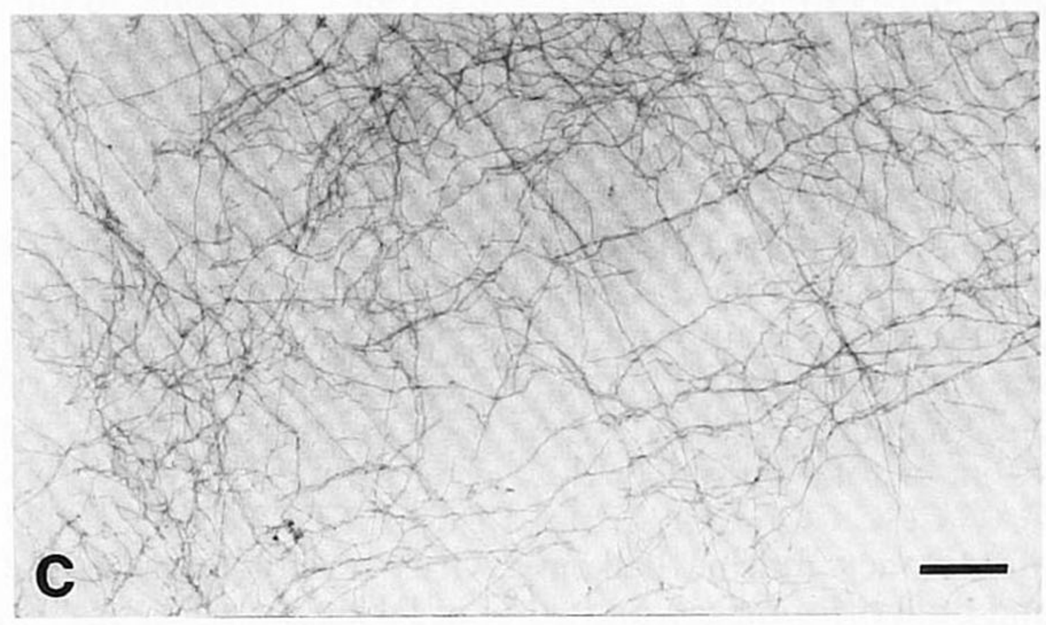


FIG. 4. Electron micrographs of negatively stained samples of actin polymerized in the presence of B-50. Actin (5 μM) was polymerized by addition of 30 mM KCl and 1 mM $MgCl_2$ in the absence (**a**) or presence of either mock-phosphorylated (**b**) or PKC-phosphorylated (**c**) B-50 (0.5 μM). Bar = 0.25 (a) or 0.30 (b and c) μm.

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