An inhibitory effect of actin on casein kinase II activity in vitro

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Abstract The inhibitory effect of actin on protein phosphorylation by three distinct protein kinases (CK-II, A-kinase and MAP-kinase) was examined in vitro. It was found that: (i) actin inhibits the activities of α-monomeric CK-II (CK-IIα) as well as oligomeric CK-II ($\alpha_2\beta_2$) in a dose-dependent manner, but has no effect on the activities of the two other kinases; and (ii) actin-induced inhibition of CK-II activity is due to the binding of actin to the α-subunit of CK-II and is non-competitive with its phosphate acceptors. In addition, it is demonstrated that actin binds directly to CK-II: both actin and CK-II are coprecipitated by anti-serum against *Drosophila* CK-IIβ or by specific IgG against *Ascaris suum* muscle actin. The results presented here suggest that actin can suppress CK-II-mediated signal transduction.

Key words: Actin; Casein kinase II; Casein kinase II activity, suppression; Rabbit muscle

1. Introduction

Casein kinase II (CK-II) is a highly conserved, cAMP-, cGMP- and Ca^{2+}-independent serine/threonine protein kinase, which has been characterized in numerous mammalian and plant cells [1,2]. CK-II is composed of three distinct subunits [α, α′ (35–42 kDa) and β (24–28 kDa)], which exist in solution as a tetramer of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ (approx. 130–150 kDa) [3]. The α- and α′-subunits, which are the products of different genes, contain the catalytic site of CK-II, whereas the β-subunit, which undergoes autophosphorylation, is responsible for regulation of CK-II activity by its direct interaction with CK-II activators, such as basic polypeptides [1,4]. Recent reports show that CK-II plays important roles in the initiation of DNA replication and transcription: it specifically modifies DNA-binding proteins (DNA topoisomerases [5] and SV-40 large T antigen [6]), transcriptional factors (Sp1 [7], serum response factor (SRF) [8] and Ap-1 [9]), some oncogene products (*erb*Aα [10], *Myb* [11] and *Myc* [12]) and suppressor gene product, p53 [13].

Recently, we reported that (i) DNA-binding sperm proteins with oligo-Arg clusters are responsible for activation of egg CK-II during fertilization in sea urchin eggs; and (ii) the protein-protein interaction between oligo-Arg and a cluster (DLEPEDELED) of acidic amino acid residues at positions 55–64 of CK-IIβ is involved in CK-II activation [14]. In our preliminary experiments concerning the purification and characterization of specific suppressive factors for CK-II in mammalian cells, a 44 kDa protein (p44), which inhibited CK-II activity in vitro, was purified from the cell extracts from brain and liver of mouse by means of protamine-affinity column chromatography. Determination of the N-terminal amino acid sequence of p44 identified the CK-II inhibitor as actin. Therefore, the present study was undertaken to characterize actin (as a potent inhibitor for CK-II activity. Here, we describe the biochemical characterization of an active actin from rabbit muscle as a CK-II inhibitor in vitro. This is the first report of such a novel in vitro inhibitory effect of actin.

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Abbreviations: CK-II, casein kinase II; A-kinase, cAMP-dependent protein kinase; C-kinase, Ca^{2+}/phospholipid-dependent protein kinase; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; MAP-kinase, mitogen-activated protein kinase; MBP, myelin basic protein; poly-Arg, poly-L-arginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]GTP (5000 Ci/mmol) and ^{125}I (100 mCi/ml) were obtained from Amersham (Arlington Heights, IL, USA); DTT, PMSF, poly-Arg, heparin, dephosphorylated α-casein (bovine milk) and myelin basic protein from Sigma Chemical Co. (St. Louis, MO, USA); histone H2B (calf thymus) from Boehringer-Mannheim Biochemicals (Germany); and murine MAP-kinase (Erk 2) from New England Biolabs Inc. (MA, USA).

2.2. Preparation of actin from rabbit skeletal muscle

Actins in acetone powder prepared from rabbit skeletal muscle were purified according to a modification of the method of Pardee and Spudich [15]. Briefly, the acetone powder (about 10 g) was suspended in 200 ml of 2 mM Tris-HCl (pH 8.0) containing 0.2 mM $CaCl_2$, 0.5 mM DTT and 0.2 mM ATP. After centrifugation (100 000 × *g* for 40 min at 4°C), actin in the supernatant was fully polymerized in 50 mM KCl containing 2 mM $MgCl_2$ and 1 mM ATP. The polymerized actin was treated with 0.6 M KCl and dialyzed for 3 days against 2 mM Tris-HCl (pH 8.0) containing 0.2 mM $CaCl_2$, 0.5 mM DTT and 0.2 mM ATP to depolymerize it. Since SDS-PAGE detected actin as a single polypeptide (approx. 44 kDa) in the dialysate, this preparation was used as a source of purified actin.

2.3. Purification of CK-II and A-kinase

Oligomeric CK-II ($\alpha_2\beta_2$) from mouse brain [16], α-monomeric CK-II from plant cells [17] and A-kinase from mouse spleen cells [18] were separately purified, as reported previously.

2.4. Assay for the activities of protein kinases

CK-II activity was measured in the standard reaction mixture (50 μl), which comprised 40 mM Tris-HCl (pH 7.6), 2 mM DTT, 3 mM Mn^{2+}, 3 μg of α-casein (phosphate acceptor), 40 μM [γ-^{32}P]ATP (500 cpm/pmol) and the indicated amounts of purified CK-II. After incubation for the indicated periods at 30°C in the presence or absence of poly-Arg (3 μg, CK-II activator), ^{32}P-labeled casein on the filter was determined with a liquid scintillation spectrometer, as reported previously [15]. A-kinase activity was measured in a reaction mixture comprising 40 mM Tris-HCl (pH 7.0), 4 mM DTT, 10 mM Mg^{2+}, 2 μM cAMP, 3 μg of histone H2B (phosphate acceptor), 40 μM [γ-^{32}P]ATP (500 cpm/pmol) and the indicated amounts of purified A-kinase. MAP-kinase activity was assayed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM Mg^{2+}, 3 μg of myelin basic protein (phosphate acceptor), 40 μM [γ-^{32}P]ATP (500 cpm/pmol) and the indicated amounts of MAP-kinase. After incubation (20 min at 30°C), ^{32}P-labeled substrates [histone H2B or myelin

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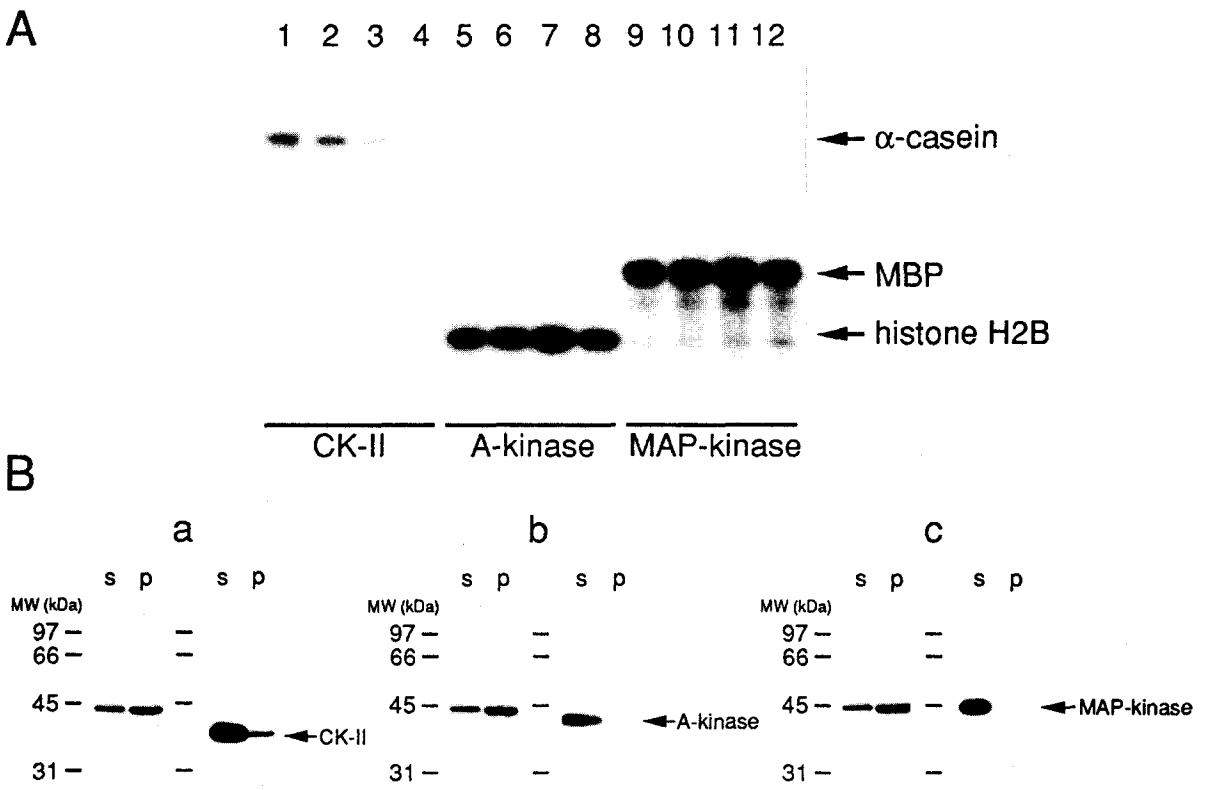

Fig. 1. (A) Effect of actin on the activities of three distinct protein kinases in vitro. The protein kinase activities were assayed separately under standard assay conditions in the presence of the indicated concentrations of actin. After incubation (20 min at 30°C) and SDS-PAGE, ^{32}P-labeled protein substrates were detected by autoradiography. Phosphorylations were α-casein (3 μg) by CK-II (approx. 0.1 μg, lanes 1–4), histone H2B (3 μg) by A-kinase (approx. 0.1 μg, lanes 5–8) and MBP (3 μg) by MAP-kinase (approx. 0.1 μg, lanes 9–12). Lanes 1,5,9, absence of actin; lanes 2,6,10, 0.4 μg of actin; lanes 3,7,11, 1.2 μg of actin; and lanes 4,8,12, 4 μg of actin. (B) Interaction of actin filaments and three distinct protein kinases. Actin (5 μg) was incubated at 4°C for 1 h in a buffer solution consisting of 40 mM Tris-HCl (pH 7.6) 2 mM Mg^{2+} and 0.1 M KCl in the presence of one of three protein kinases (a, CK-II; b, A-kinase; and c, MAP-kinase). After centrifugation at 100 000×g for 1 h at 2°C, the catalytic subunits of these three kinases in the supernatants (s) and precipitates (p) obtained were detected by the active gel method [20].

basic protein (MBP)] were determined by SDS-PAGE followed by autoradiography.

2.5. SDS-PAGE and autoradiography

SDS-PAGE followed by autoradiography to detect ^{32}P-labeled polypeptides was performed according to method originally described by Laemmli [19], after protein phosphorylation by the indicated protein kinases, as reported in [14].

2.6. Detection of the catalytic subunits of protein kinases

The catalytic subunits of protein kinases on the electrophoresis gel were detected by a modification of a method described originally by Kameshita and Fujisawa [20]. The gel was washed twice with buffer [50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol (2-ME)] containing 20% 2-propanol, and then rinsed in the same buffer for 1 h at room temperature. The subunits of protein kinases were renatured with 6 M guanidine-HCl in the same buffer for 1 h at room temperature, and then renatured with the same buffer containing 0.04% Tween 40 for 18 h at 4°C. After renaturation, protein phosphorylation was performed by incubation of the gel in 2.5 ml of a buffer [40 mM HEPES-NaOH (pH 8.0), 2 mM 2-ME and 3 mM Mn^{2+}] containing 20 μM [γ-^{32}P]ATP (2000 cpm/pmol) for 1 h at room temperature. After washing the gel extensively with 5% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate, the ^{32}P-labeled catalytic subunits of protein kinases on the gel were detected by autoradiography.

3. Results

3.1. Selective inhibitory effect of actin on CK-II activity in vitro

To characterize the inhibitory effect of actin from rabbit skeletal muscle on the activities of three distinct serine/threonine protein kinases (CK-II, A-kinase and MAP-kinase), they were separately assayed in the presence of actin at the indicated doses in vitro. It was found that actin inhibited CK-II activity in a dose-dependent manner, but did not affect the activities of the two other kinases (Fig. 1A).

To confirm the direct interaction between actin and these three kinases, it was incubated separately with each kinase under conditions conducive to actin polymerization. After separation of polymerized actin by centrifugation, the catalytic subunits of the three kinases in the supernatant and the precipitate fractions were detected by the active gel method [20]. As shown in Fig. 1B, CK-II activity was detected in the precipitate fraction of the polymerized actin, whereas the catalytic subunits of the two other kinases (A-kinase and MAP-kinase) were detected only in the supernatants. These results suggest that, among the three kinases tested, only CK-II exhibits complex formation on incubation with actin in vitro.

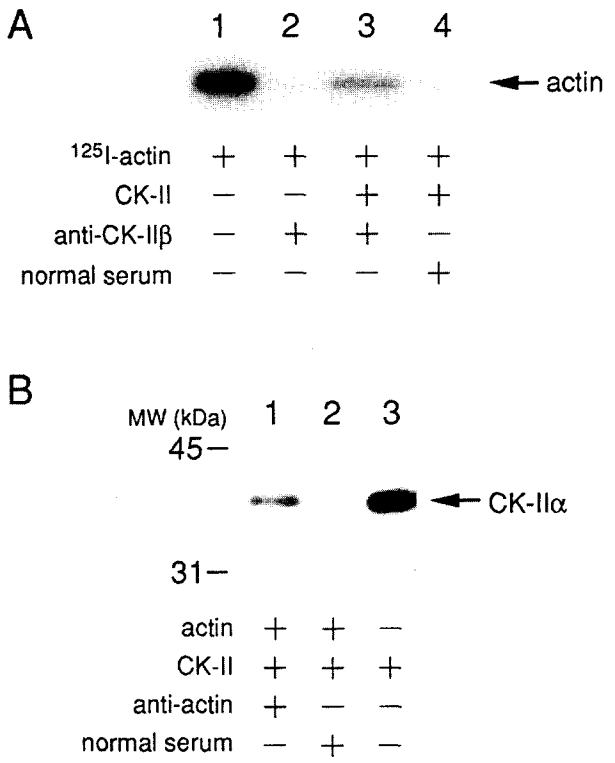

Fig. 2. (A) Coimmunoprecipitation of actin and oligomeric CK-II with anti-CK-IIβ antibody in vitro. ^{125}I-labeled actin and oligomeric CK-II (approx. 0.1 μg) were incubated for 12 h at room temperature with either rabbit anti-serum against *Drosophila* CK-IIβ antibody (lane 3) or normal rabbit serum (lane 4). After incubation for a further 2 h in the presence of protein A-Sepharose, the resulting immunoprecipitates were washed four times with buffer [10 mM Tris-HCl (pH 7.6), 0.15 M NaCl and 1% NP-40], and then subjected to SDS-PAGE followed by autoradiography. Lane 1, ^{125}I-labeled actin as a control; lane 2, ^{125}I-labeled actin incubated for 12 h at room temperature with anti-CK-IIβ antibody in the absence of oligomeric CK-II. (B) Direct binding of oligomeric CK-II to actin in vitro. Purified actin (approx. 2 μg) and oligomeric CK-II (approx. 0.1 μg) were incubated for 1 h at room temperature, and then incubated for 12 h with either rabbit anti-IgG against *Ascaris suum* muscle actin [21] or normal rabbit serum. After incubation for a further 2 h in the presence of protein A-Sepharose, the resulting immunocomplexes were washed four times with washing buffer [10 mM Tris-HCl (pH 7.6), 0.15 M NaCl and 1% NP-40], and subjected to the active gel phosphorylation assay [20] to detect the α-catalytic subunit of CK-II (CK-IIα). Actin and CK-II were incubated with anti-IgG against *Ascaris suum* muscle actin (lane 1) or normal rabbit serum (lane 2) in the presence of protein A-Sepharose. Lane 3, mouse CK-II ($\alpha_2\beta_2$) as a control.

3.2. Direct binding of actin to CK-II

The above experimental result that CK-II was coprecipitated with polymerized actin was further confirmed by immunological experiments. To investigate the binding of actin to CK-II, ^{125}I-labeled actin was pre-incubated for 12 h at room temperature with oligomeric CK-II ($\alpha_2\beta_2$), and then incubated for 2 h at 4°C with anti-serum against *Drosophila* CK-IIβ in the presence of protein A-Sepharose. As expected, the antiserum precipitated a radioactive immunocomplex only in the presence of both ^{125}I-labeled actin and CK-II (Fig. 2A). Similar precipitation of actin and oligomeric CK-II was also observed on incubation with specific IgG against *Ascaris suum* muscle actin in the presence of protein A-Sepharose (Fig. 2B). These two observations confirm that actin binds directly to CK-II.

3.3. Characterization of actin as a CK-II inhibitor

It is well known that CK-II utilizes both ATP and GTP as phosphate donors [1], whereas only ATP binds to actin [22]. Therefore, the inhibitory effect of actin on CK-II activity (phosphorylation of α-casein) was examined using ATP or GTP as a phosphate donor. As shown in Fig. 3A, actin inhibits CK-II activity when either ATP or GTP is used as a phosphate donor. Since actin as well as CK-II bound to poly-Arg, the inhibitory effect of actin on CK-II activity was assessed in the presence or absence of poly-Arg. As expected, actin-induced inhibition of CK-II activity was still observed when assayed in the absence of poly-Arg (Fig. 3B). In addition, similar inhibition of CK-II activity by actin was observed when other CK-II phosphate acceptors, such as 94 kDa glucose regulated protein (GRp94) or calreticulin (Ca^{2+}-binding protein), were used instead of α-casein under the same experimental conditions (data not shown).

To determine the direct effect of actin on the α-subunit (catalytic subunit) of CK-II, α-monomeric CK-II (CK-IIα) purified from plant cells [17] was used. As expected, the CK-IIα-catalyzed phosphorylation of α-casein was inhibited by actin in a dose-dependent manner (Fig. 4A). Under the given experimental conditions, phosphorylation of α-casein by CK-IIα increased linearly up to 20 min at 30°C (Fig. 4B). This phosphorylation was inhibited completely when ac-

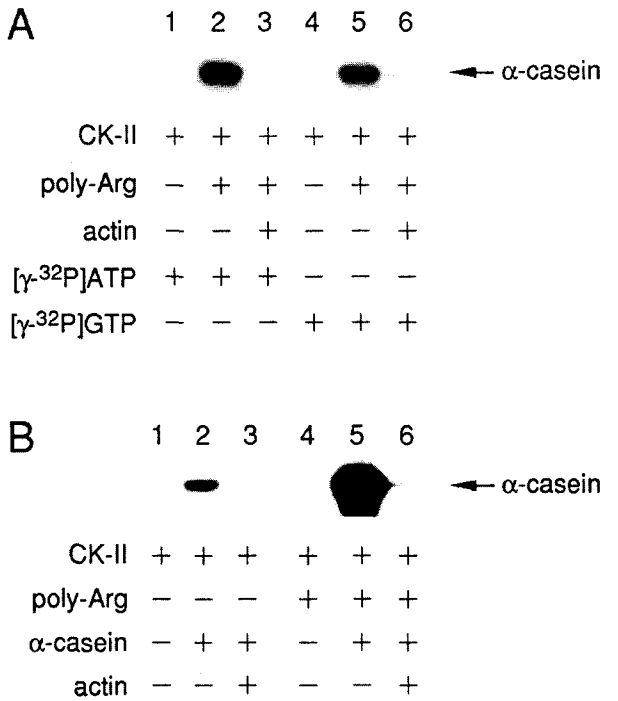

Fig. 3. (A) Inhibitory effect of actin on CK-II activity. Phosphorylation of α-casein by CK-II was carried out using 40 μM [γ-^{32}P]ATP (500 cpm/pmol; lanes 1–3) or 40 μM [γ-^{32}P]GTP (500 cpm/pmol; lanes 4–6) as a phosphate donor under standard assay conditions. Lanes: 1,4, CK-II (0.1 μg) incubated for 20 min at 30°C with α-casein in the absence of poly-Arg (3 μg) and actin (4 μg); 2,5, in the presence of poly-Arg (3 μg); 3,6, in the presence of poly-Arg (3 μg) and actin (4 μg). (B) Inhibitory effect of actin on CK-II activity in the presence or absence of poly-Arg. To examine the effect of poly-Arg, CK-II (0.1 μg) was incubated for 20 min at 30°C with α-casein (3 μg), actin (4 μg) and 40 μM [γ-^{32}P]ATP (500 cpm/pmol) in the absence (lanes 1–3) or presence (lanes 4–6) of poly-Arg (3 μg). ^{32}P-labeled α-casein in the reaction mixtures was detected by autoradiography after SDS-PAGE.

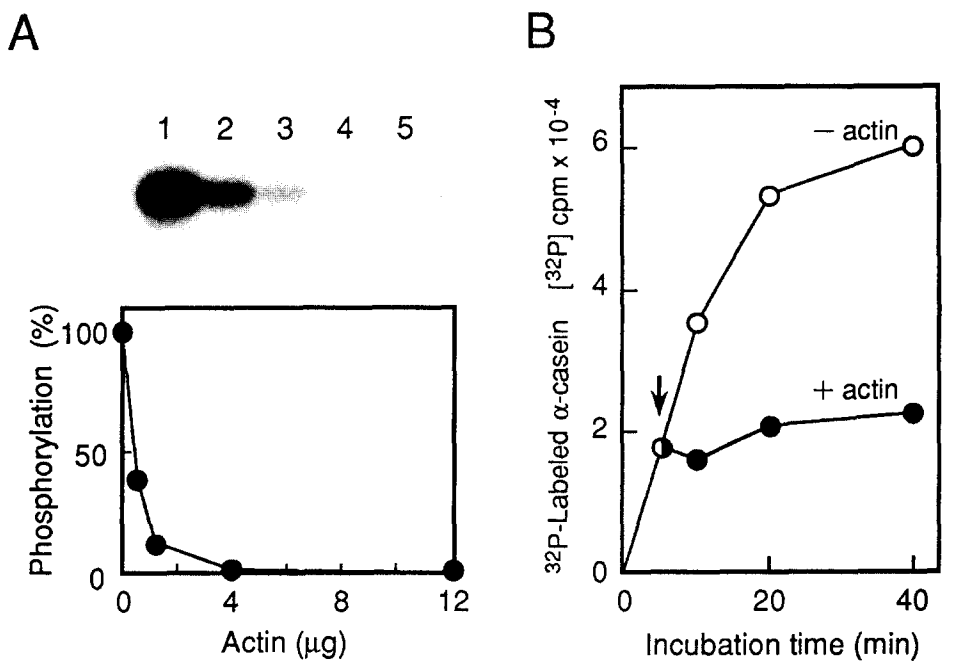

Fig. 4. (A) Dose-effect of actin on phosphorylation of α-casein by α-monomeric CK-II (CK-IIα). CK-IIα activity was assayed under standard assay conditions in the presence of the indicated concentrations of actin, using α-casein as a phosphate acceptor. After incubation (20 min at 30°C) in the absence of poly-Arg, ^{32}P-labeled α-casein was detected by autoradiography after SDS-PAGE. Lanes: 1, phosphorylation of α-casein by CK-IIα in the absence of actin; 2, lane 1+0.4 μg of actin; 3, lane 1+1.2 μg of actin; 4, lane 1+4 μg of actin; 5, lane 1+12 μg of actin. The autoradiogram was scanned by spectrophotometry and the inhibitory effect of actin represented as a percentage of that determined in the absence of actin (●). (B) Effect of actin on phosphorylation of α-casein by CK-IIα. Phosphorylation of α-casein by CK-IIα was examined at 30°C under standard assay conditions. Purified actin (4 μg) was added to the reaction mixture at 5 min after the initiation of protein phosphorylation by addition of 40 μM [γ-^{32}P]ATP (500 cpm/pmol) (●). Phosphorylation of α-casein by CK-IIα was also allowed to proceed in the absence of actin (○).

tin was added to the reaction mixtures at 5 min after incubation of CK-IIα with 40 μM [γ-^{32}P]ATP (500 cpm/pmol) at 30°C. These results suggest that the actin-induced inhibition of CK-II activity is due to the binding of actin to the α-subunit of CK-II in vitro.

3.4. Kinetics of CK-II inhibition by actin in vitro

To characterize the kinetics of the actin-induced inhibition of CK-II activity, α-casein was used as a phosphate acceptor. As shown in Fig. 5, there was a substantial alteration in V_{max} from 0.74 to 0.31 pmol min^{-1} when actin (approx. 0.46 μM) was added to the reaction mixtures. However, no change of K_m (1.1 μM) for the substrate was detected under the given experimental conditions. A similar inhibitory effect of actin against CK-II activity was observed when other CK-II substrates, such as GRp94 and myosin heavy chain, were used instead of α-casein as the phosphate acceptor for CK-II (data not shown). These results indicate that, with CK-II substrates, actin inhibits the activity of oligomeric CK-II ($\alpha_2\beta_2$) in a non-competitive manner.

4. Discussion

Muscle-type actin functions as a potent inhibitor for CK-II in vitro. This conclusion is supported by the following observations: (i) actin inhibits CK-II activity in a dose-dependent manner, but does not affect two other kinases (A-kinase and MAP-kinase; Fig. 1); (ii) actin-induced inhibition is reproduced when [γ-^{32}P]GTP is used instead of [γ-^{32}P]ATP as a phosphate donor for CK-II (Fig. 3A); (iii) actin inhibits CK-II activity through its direct binding to the α-subunit of CK-II in vitro (Fig. 4A); and (iv) both CK-II and actin can be coprecipitated with anti-serum against *Drosophila* CK-IIβ or specific IgG against *Ascaris suum* muscle actin (Fig. 2). In addition, the actin-induced inhibition of CK-II activity was further characterized as being non-competitive with α-casein as a phosphate acceptor (Fig. 5). However, no significant effect of actin on CK-II activity was observed when actin was inactivated by treatment with 0.5 M NaCl containing 1 mM EDTA and 0.5 mM EGTA. No inhibition of CK-II activity was detected when fragments generated from actin (after its limited digestion with modified trypsin) were incubated with CK-II and α-casein in vitro.

Actins are abundant gene products, ubiquitously expressed in all eukaryotic cells [22], with highly conserved amino acid sequences among mammalian species and only 17 of the 375 amino acid residues of human actin substituted with other amino acids in the actin sequence of *Physarum polycephalum* [21]. In vertebrates, there are at least six actin isoforms: four muscle types (skeletal muscle, cardiac, aorta-type smooth muscle and stomach-type smooth muscle actins) and two non-muscle types (cytoplasmic β- and γ-actins) [23]. Muscle-type actins are tissue specific and functionally involved in muscle contractions, whereas cytoplasmic actins are expressed in many kinds of cell and participate in a variety of cell functions [24]. It seems that all types of actin may be responsible for various cell functions since more than 30 actin-binding proteins have been identified and characterized biochemically [22]. There is, probably, a common ability to inhibit the CK-II-catalyzed phosphorylation of intracellular CK-II substrates,

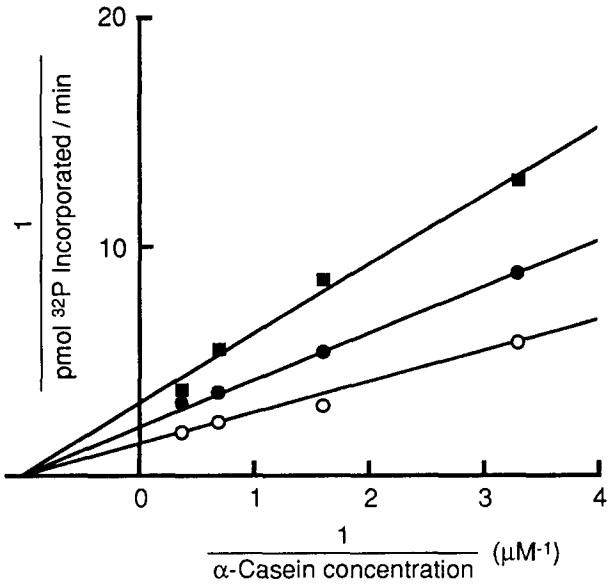

Fig. 5. Kinetics of actin-induced inhibition of CK-II activity. Double-reciprocal plots of inhibition by actin against CK-II activity were constructed. CK-II activity was assayed under standard conditions (10 min at 30°C) using α-casein as a phosphate acceptor in the presence or absence of actin. ^{32}P-labeled α-casein was detected with a liquid scintillation spectrometer. CK-II (approx. 0.1 μg) incubated with α-casein: (○—○) in the absence of actin (control); (●—●) in the presence of 0.23 μM actin; or (■—■) 0.46 μM.

since muscle-type actins from rabbit muscle (Fig. 5), hen muscle and mouse brain inhibit CK-II activity in a dose-dependent manner without species specificity.

Earlier reports concerning the regulatory mechanisms of CK-II activity in mammalian cells have demonstrated that (i) CK-II activity is regulated through its phosphorylation by p34^{cdc2} kinase [25], protein kinase C [26] or autophosphorylation of CK-IIβ [27]; (ii) nuclear calmodulin inhibits significantly the phosphorylation of at least three major nuclear proteins (100, 42–44 and 37 kDa) by CK-II in the nuclear fraction of rat liver in vitro [28]; and (iii) the interaction between DNA and CK-IIα results in inhibition of CK-II activity in vitro [29]. However, the suppressive mechanisms of CK-II activity by these two cellular components (calmodulin and DNA) at the cellular level and their biological significances in the regulatory mechanisms involved in transcription and DNA replication remain to be elucidated. Our finding that actin acts as a potent inhibitor for CK-II in vitro suggests that there is a further dimension of CK-II-mediated alteration of metabolic pathways and transcriptional regulation in differentiating or proliferating cells. This speculation is supported by evidence that (i) CK-II is characterized as one of the kinases responsible for metabolic alteration through phosphorylation of a number of enzymes and cellular proteins during cell differentiation and proliferation [1,2]; and (ii) CK-II plays an important role in positive and negative transcriptional regulations through specific phosphorylation of various transcriptional factors by the kinase [1,7–9], as mentioned in Section 1. However, to understand clearly the biological significance of actin in the regulation of CK-II-mediated signal transduction, the following fundamental questions remain to be elucidated through further analytical studies: (i) the biochemical mechanisms of actin involvement in CK-II-mediated signal transduction during cell proliferation and differentiation; and (ii) the physiological correlation between CK-II, actin and other actin-associated protein kinases in mammalian cells.

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