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Protein kinase C and cAMP-dependent protein kinase induce opposite effects on actin polymerizability

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Protein kinase C phosphorylated muscle and non-muscle monomeric actin more efficiently than filamentous actin in vitro. By sedimentation assay, the ratio of phosphorylated to unphosphorylated actin was much higher in sedimentable actin than in the non-sedimentable form, suggesting that phosphorylated actin was more readily incorporated into F-actin than unphosphorylated actin. In contrast, actin phosphorylated by cAMP-dependent protein kinase was found to have weaker polymerizability than the unphosphorylated form. The phosphopeptide mapping pattern of actin phosphorylated by protein kinase C was different from that of actin phosphorylated by cAMP-dependent protein kinase. Thus, both the protein kinases phosphorylate actin differently and induce opposite effects on actin polymerizability.

Protein kinase C; Actin; cyclic AMP-dependent protein kinase; Cytoskeleton; Signal transduction

1. INTRODUCTION

Much attention has been paid to the turnover of inositol phospholipids in the membrane and subsequent activation of protein kinase C [1] which has been generally regarded as the receptor for tumor-promoting phorbol esters such as TPA [2]. TPA causes dramatic reorganization of microfilaments [3], suggesting that activation of protein kinase C regulates actin polymerization in living cells. It has previously been reported that several actin-related cytoskeletal proteins such as myosin [4,5], vinculin [6–8], filamin [8], troponin [9], talin [10] and myosin light chain kinase [11] serve as substrates for protein kinase C. However, phosphorylation of actin itself by protein kinase C has not been studied well. Here, we show that protein kinase C phosphorylates actin and alters its polymerizability. In addition, we show further that phosphorylation of actin by cAMP-dependent protein kinase induces opposite effects on actin polymerizability.

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2. MATERIALS AND METHODS

Protein kinase C was purified from the soluble fraction of rabbit brain as in [12]. Actins from rabbit skeletal muscle [13], porcine brain [14] and rat ascites hepatoma (AH7974) [15] were prepared as described. [γ-^{32}P]ATP was purchased from ICN Radiochemicals. Catalytic subunit of cAMP-dependent protein kinase from bovine heart was obtained from Sigma. Phosphorylation of actin by protein kinase C was performed in a reaction mixture (50 μl) which contained 5 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 0.4 mM EGTA, 0.8 mM $CaCl_2$, 40 mM KCl, 40 μM [γ-^{32}P]ATP and 0.1 μg protein kinase C with or without 4 μg phosphatidylserine and 0.04 μg diolein. Phosphorylation of actin by the catalytic subunit of cAMP-dependent protein kinase was carried out in a reac-

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tion mixture (50 μl) which contained 7 mM Pipes (pH 7.2), 70 mM KCl, 5 mM $MgCl_2$, 6 mM DTT, 20 μM [γ-^{32}P]ATP and 0.75 μg catalytic subunit of cAMP-dependent protein kinase. The reaction was started by the addition of 5 μg G-actin or F-actin. After incubation for appropriate intervals at 30°C, an aliquot was mixed with 1/3 vol. of an SDS stop solution and boiled for 2 min. The sample was subjected to SDS-PAGE according to Laemmli [16], and the actin band was cut out of the gel to measure the radioactivity in a liquid scintillation counter. Two-dimensional gel electrophoresis was performed by the method of O'Farrell et al. [17]. Analysis of phosphorylated amino acids was performed as in [11]. Peptide mapping was carried out according to Cleveland et al. [18]. The polymerizability of phosphorylated actin was assayed by centrifugation followed by SDS-PAGE and autoradiography. G-Actin (110 μg/ml) or F-actin (110 μg/ml) was mixed with protein kinase C or the catalytic subunit of cAMP-dependent protein kinase in a solution of 2 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 2 mM $CaCl_2$, 2.5 mM DTT, 50 μM [γ-^{32}P]ATP, with or without 5 μg phosphatidylserine and 0.05 μg diolein, and incubated for 1 h at 30°C. In some experiments, the mixture was diluted 6-fold with a low ionic strength buffer solution of 2 mM Hepes and 0.1 mM DTT (pH 7.8) and further incubated for 40 min at 30°C. These samples were centrifuged at 100000 × g for 40 min at 20°C and the supernatant and the pellet fractions were subjected to SDS-PAGE. The gels were stained with Coomassie brilliant blue, and the intensity of the actin bands was determined by scanning the gels in a densitometer. The actin bands were then cut out of the gels and the radioactivity was determined in a scintillation counter.

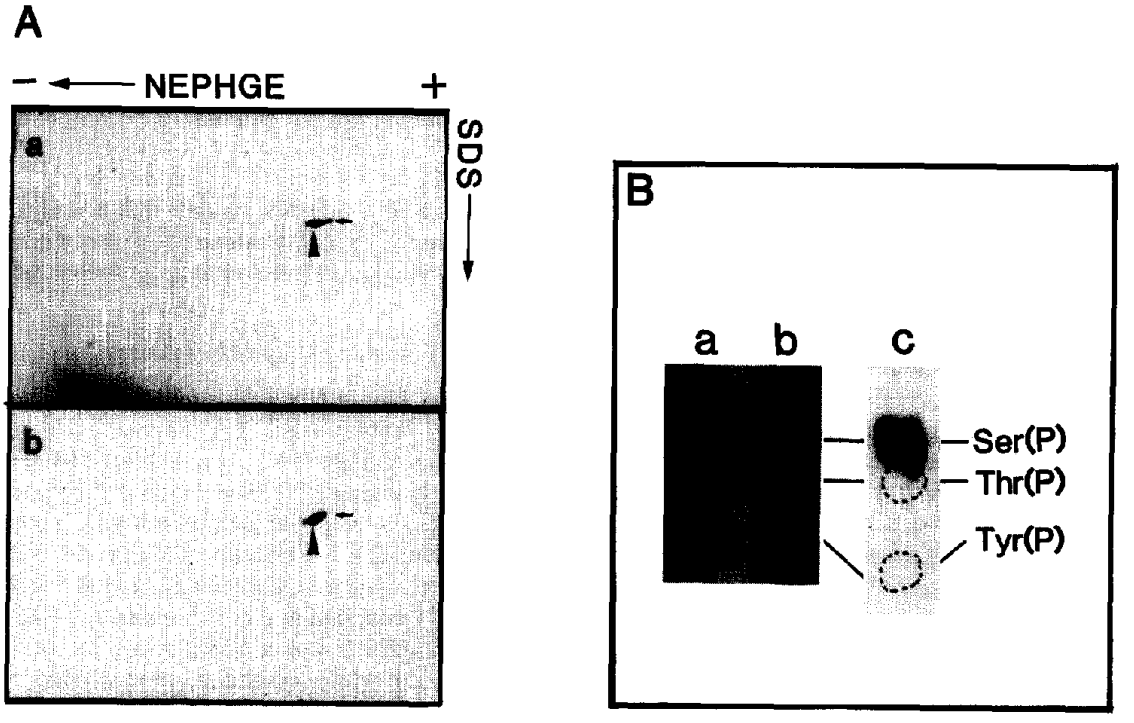

Fig.1. (A) Two-dimensional gel electrophoresis of actin after phosphorylation by protein kinase C. Brain actin was phosphorylated as described in section 2 for 20 min and processed for two-dimensional NEPHGE/SDS-PAGE. (a) Coomassie blue staining, (b) autoradiograph. Positions of the arrowhead and arrow in (a) correspond to those in (b). (B) Phosphoamino acid analysis of actin phosphorylated by protein kinase C (a,b) or cAMP-dependent protein kinase (c). Phosphorylation was carried out for 20 min. The positions of phosphoserine, phosphothreonine and phosphotyrosine are indicated. a,c, muscle actin; b, brain actin.

3. RESULTS

Porcine brain actin was found to serve as a substrate for the purified protein kinase C (fig.1A). The phosphorylation was dependent on the presence of Ca^{2+}, phosphatidylserine and diolein (not shown). After incubation for 20 min at 30°C in the presence of 20 μM ATP, about 0.03 mol phosphate was incorporated into 1 mol actin. Two-dimensional gel electrophoresis confirmed that the phosphorylated protein was in fact actin (fig.1A). Rabbit skeletal muscle actin was also phosphorylated by the purified protein kinase C to the same extent as in brain actin (not shown). Fig.1B shows that the phosphorylation occurred mainly on serine and slightly on threonine residues in both muscle and non-muscle actins.

To investigate which form of actin, G-actin or F-actin, is the better substrate for protein kinase C, we increased the G-actin concentration by adding barbed end-capping protein, 88-kDa protein/actin complex (0.04 μM) [19], to the F-actin solution (1.0 μM). This resulted in protein kinase C phosphorylating actin 2.2-fold more rapidly in the presence of the complex (743 cpm) than in its absence (338 cpm). Moreover, phosphorylation of actin was more rapid when the reaction was started by the addition of G-actin than by the addition of preformed F-actin (table 1). These results strongly indicate that G-actin is more readily phosphorylated by protein kinase C than F-actin.

We found that the phosphorylated actin was polymerized into F-actin more efficiently than unphosphorylated actin. Table 1 shows that the ratio of phosphorylated to unphosphorylated actin was much higher in sedimented than non-sedimented actin. This was observed irrespective of whether phosphorylation occurred during polymerization

Table 1

Effect of actin phosphorylation by protein kinase C (I) or cAMP-dependent protein kinase (II) on actin polymerizability

	^{32}P in actin cpm (%)		Total actin μg/ml (%)	
	Sup	Ppt	Sup	Ppt
I. Protein kinase C				
A (a)	50 (1.8)	2675 (98.2)	20.0 (20.0)	80.0 (80.0)
(b)	25 (2.0)	1200 (98.0)	19.0 (19.0)	81.0 (81.0)
B	200 (16.7)	1000 (83.3)	8.8 (48.1)	9.5 (51.9)
II. cAMP-dependent protein kinase				
A (a)	625 (21.4)	2300 (78.6)	5.0 (4.5)	105 (95.5)
(b)	175 (16.7)	875 (83.3)	4.0 (3.6)	106 (96.4)
B	1440 (61.7)	893 (38.3)	5.3 (29.0)	13.0 (71.0)

(A) Protein kinase C (I-A) or the catalytic subunit of cAMP-dependent protein kinase (II-A) was incubated with actin (brain actin 100 μg/ml, I-A; muscle actin 110 μg/ml, II-A) during polymerization of actin (a) or after polymerization had reached the plateau (b) as described in section 2. Polymerized (ppt) and unpolymerized (sup) actin were then separated by centrifugation followed by SDS-PAGE and the ^{32}P radioactivity and actin content of each fraction were determined. (B) Actin (muscle actin 110 μg/ml) was phosphorylated by protein kinase C (I-B) or by the catalytic subunit of cAMP-dependent protein kinase (II-B) as in A (a). The samples were then diluted 6 times in a low ionic strength solution and further incubated for 40 min at 30°C. The polymerized (ppt) and unpolymerized (sup) actin were separated by centrifugation and processed as described in (A)

[table 1, IA(a)] or after polymerization [table 1, IA(b)]. When the F-actin was diluted in a low ionic strength solution to induce partial depolymerization, part of the phosphorylated actin also depolymerized and became non-sedimentable. However, the phosphorylated actin was still enriched in the sedimented actin fraction (table 1, IB). The phosphorylated actin was mostly eluted together with the bulk of F-actin and partly with monomeric actin on a Sepharose CL-6B column (not shown), further suggesting that the phosphorylated actin is more readily incorporated into F-actin than the unphosphorylated form.

Neither type II Ca^{2+}/calmodulin-dependent protein kinase nor epidermal growth factor receptor kinase phosphorylated muscle actin under our experimental conditions. However, the catalytic subunit of cAMP-dependent protein kinase from bovine heart phosphorylated muscle actin and brain actin. After incubation for 20 min at 30°C in the presence of 20 μM ATP, about 0.05 mol phosphate was incorporated into 1 mol actin. The rates of incorporation of phosphate to non-muscle (brain) actin and muscle actin were approximately the same (not shown). Phosphorylation occurred exclusively on serine residues in muscle actin (fig.1C). Table 1,IIA shows that the phosphorylation of actin was more rapid when the reaction was started by the addition of G-actin, suggesting that G-actin is a better substrate than F-actin, as in phosphorylation by protein kinase C. However, in contrast to actin phosphorylated by protein kinase C, actin phosphorylated by cAMP-dependent protein kinase was found to have a weaker affinity for F-actin than unphosphorylated actin (table 1,IIA), namely the ratio of phosphorylated to unphosphorylated actin was much lower in the sedimented than non-sedimented actin. When the F-actin was diluted in a low ionic strength solution, more than 60% of the phosphorylated actins were depolymerized while 70% of the unphosphorylated actins were still polymerized under the conditions used (table 1,IIB).

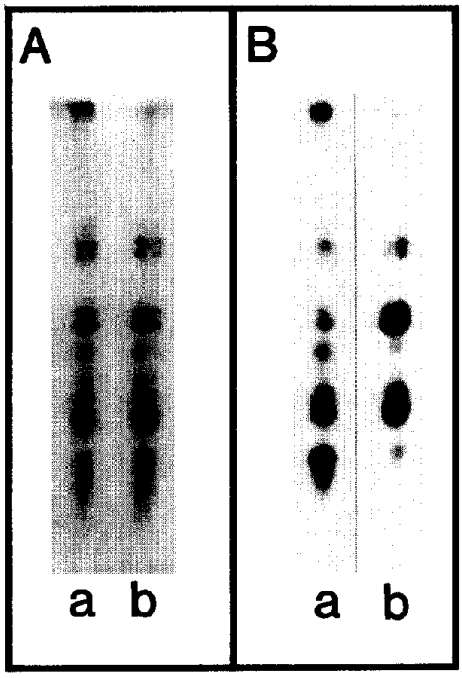

Fig.2. Phosphopeptide maps of muscle actin phosphorylated by cAMP-dependent protein kinase (a) and by protein kinase C (b). Each phosphorylation reaction was carried out for 20 min at 30°C. Phosphopeptide mapping was performed with 20 ng V8 protease. (A) Coomassie blue staining pattern of actin fragments, (B) autoradiograph.

The phosphopeptide mapping revealed that the preferential phosphorylation sites in actin are different between the protein kinase C and cAMP-dependent protein kinase (fig.2). This suggests that the two kinases phosphorylate mainly different site(s) on actin molecule.

4. DISCUSSION

We have shown that protein kinase C phosphorylates muscle and non-muscle actin and increases their polymerizability. We have also found that the catalytic subunit of cAMP-dependent protein kinase phosphorylates both muscle and non-muscle actin and decreases their polymerizability. The phosphopeptide mapping revealed that the major phosphorylated peptides of actin are different between protein kinase C and cAMP-dependent protein kinase. The phosphoamino acid analysis showed that protein kinase C phosphorylated both serine and threonine residues in actin, whereas cAMP-dependent protein kinase phosphorylated only serine residues. These results strongly suggest that both the kinases differently phosphorylate actin and induce opposite effects on actin polymerizability.

Walsh et al. [20] previously showed that smooth and skeletal actins were phosphorylated by cAMP-dependent protein kinase, although they did not investigate the polymerizability of the phosphorylated actin. Grazi and co-workers [21,22] have shown that skeletal G-actin was phosphorylated upon incubation with liver plasma membrane and lost its ability to polymerize, although the kinase responsible for phosphorylation was not identified. Furthermore, Sonobe et al. [23] reported that *Amoeba* actin was phosphorylated by an unknown endogenous kinase and lost its polymerizability. The present study has clearly shown that cAMP-dependent protein kinase-mediated phosphorylation of actin decreases its polymerizability. In contrast, the protein kinase C-mediated increase in actin polymerizability found here is very intriguing. It is unlikely that actin phosphorylated by protein kinase C makes aggregates via irreversible denaturation, because at least part of the phosphorylated actin depolymerized with unphosphorylated actin when the F-actin was diluted in a low ionic strength solution. Moreover, the gel filtration experiment showed that the phosphorylated actin mostly acted with the bulk of the unphosphorylated actin filaments. These results may suggest that phosphorylated actin makes random copolymers with the unphosphorylated actin. Considering that *Physarum* actin filament capping proteins are structurally related to actin itself [19], actin phosphorylated by protein kinase C might have some structural and functional similarities to these capping proteins especially in that they all have a stronger affinity to F-actin.

Protein kinase C is regarded as the receptor for tumor-promoting phorbol esters such as TPA which causes dramatic reorganization of microfilaments in cultured cells [3]. Furthermore, one of the early responses stimulated by platelet-derived growth factor or epidermal growth factor is polymerization of actin, causing an outgrowth of microspikes and ruffling membranes [25,26]. Both platelet-derived growth factor [27] and epidermal growth factor [28] increase phosphatidylinositol turnover and possibly induce activation of protein kinase C. In these cases, actin phosphorylation by protein kinase C may play a role in reorganization of actin filaments.

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