# Site-Specific Phosphorylation by Protein Kinase C Inhibits Assembly-Promoting Activity of Microtubule-Associated Protein 4†

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ABSTRACT: We have examined the phosphorylation of bovine microtubule-associated protein 4 (MAP4), formerly named MAP-U, by protein kinase C (PKC). When MAP4 was incubated with PKC, about 1 mol of phosphate was incorporated/mol of MAP4. Phosphorylation of MAP4 caused a remarkable decrease in the ability of the MAP to stimulate microtubule assembly. MAP4 consists of an amino-terminal projection domain and a carboxyl-terminal microtubule-binding domain. The carboxyl-terminal domain is subdivided into a Pro-rich region and an assembly-promoting (AP) sequence region containing four tandem repeats of AP sequence that is conserved in MAP4, MAP2, and $\tau$ [Aizawa et al. (1990) *J. Biol. Chem. 265*, 13849–13855]. In order to identify the site of MAP4 phosphorylated by PKC, a series of expressed MAP4 fragments was prepared and treated with the kinase. A fragment corresponding to the Pro-rich region (P fragment) was phosphorylated, while fragments corresponding to the projection domain and the AP sequence region were not. In addition, chymotryptic digestion of an authentic MAP4 prephosphorylated by PKC revealed that phosphate was incorporated almost exclusively into a 27-kDa fragment containing the carboxyl-terminal half of the Pro-rich region. We investigated the phosphorylation site in MAP4 using the P fragment and found that $Ser^{815}$ was phosphorylated almost exclusively. We conclude that the phosphorylation of a single Ser residue in the Pro-rich region negatively regulates the assembly-promoting activity of MAP4.

Microtubule-associated proteins (MAPs)[1] is the general term for the nontubulin components of microtubules. MAPs have the ability to stimulate tubulin polymerization and to bind to reconstituted microtubules in vitro (Weingarten et al., 1975; Murphy & Borisy, 1975; Dustin, 1984). In contrast to tubulin, whose amino acid sequence is conserved among eukaryotic cells, MAPs show structural diversity and are believed to confer upon microtubules a variety of structures and functions (Vallee et al., 1984). Among MAPs, MAP1, MAP2, $\tau$, and MAP4 have been studied and characterized in detail (Cleveland et al., 1977; Kim et al., 1979; Kuznezov et al., 1981; Parysek et al., 1984; Murofushi et al., 1986). MAP1, MAP2, and $\tau$ exist predominantly in brain (Huber & Matus, 1984; Binder et al., 1985; Lewis et al., 1986; Goedert et al., 1988), while MAP4 is distributed ubiquitously as a major MAP among mammalian tissues and cells [bovine MAP-U (Murofushi et al., 1986), rat 190-kDa MAP (Kotani et al., 1988), murine MAP4 (Parysek et al., 1984), human HeLa MAP (Bulinski & Borisy, 1980; Weatherbee et al., 1980)].

In terms of primary structure, bovine MAP4 can be divided into an N-terminal projection domain and a C-terminal microtubule-binding domain (Aizawa et al., 1990; see Figure 2). The microtubule-binding domain is composed of a Pro-rich region, an assembly-promoting (AP) sequence region, and a hydrophobic C-terminal tail region. The AP sequence region is common among heat-stable MAPs (MAP4, MAP2, $\tau$) (Lee et al., 1988; Lewis et al., 1988; Himmler et al., 1989; Aizawa et al., 1990) and has been shown to play a central role in the assembly of morphologically normal microtubules (Aizawa et al., 1989). Recently, we have also shown that the Pro-rich region confers a high affinity for microtubules upon MAP4 (Aizawa et al., 1991).

Now that the structure–function correlation in MAP4 has been basically elucidated, growing interest is focused on the regulation of its activity. In studies of the regulatory mechanism of MAP activity, it was demonstrated that phosphorylation of neural MAPs, MAP2 and $\tau$, by protein kinase C (PKC) (Hoshi et al., 1987, 1988), cyclic AMP dependent protein kinase (Burns et al., 1984), or unidentified kinases copurified with microtubule proteins (Lindwell & Cole, 1984) reduced their assembly-promoting activity. However, details of the phosphorylated sites have not been elucidated. Furthermore, few data have been published on the phosphorylation of nonneural MAPs that may participate in the regulation of microtubule stability in nonneural cells. In this paper, we report the phosphorylation by PKC of MAP4, a major MAP in various kinds of cells. We show that phosphorylation suppresses the ability of MAP4 to promote microtubule assembly in vitro and that the site phosphorylated by PKC is a specific Ser residue in the Pro-rich region, a part of the

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[1] Abbreviations: MAPs, microtubule-associated proteins; AP, assembly promoting; PS, phosphatidylserine; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; LEP, lysylendopeptidase; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; **RB**, reassembly buffer (100 mM Pipes, 2 mM EGTA, and 1 mM $MgCl_2$); HPLC, high-performance liquid chromatography.

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microtubule-binding domain of MAP4.

## Materials and Methods

*Chemicals.* Bovine brain phosphatidylserine (PS) was a product of Serdary Research Laboratories (Ontario, Canada). Phenylmethanesulfonyl fluoride (PMSF), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), chymotrypsin (type VII), and the catalytic subunit of cyclic AMP dependent protein kinase from bovine heart were products of Sigma (St. Louis, MO). [$\gamma$-$^{32}$P]ATP was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Lysylendopeptidase (LEP) was purchased from Wako (Osaka, Japan). Casein kinase II purified from porcine testis was generously supplied by Dr. S. Nakajo, Showa University, Tokyo. Taxol was generously supplied by Dr. N. Lomax, National Cancer Institute, Bethesda, MD. All other materials were of reagent grade.

*Preparation of Proteins.* Bovine brain tubulin was prepared from three-cycled microtubule proteins (Shelanski et al., 1973) by the method of Weingarten et al. (1975). MAP4 was purified from bovine adrenal cortex according to the method of Murofushi et al. (1986). Conventional PKC was purified from rabbit brains by the method of Kitano et al. (1986). MAP4 fragments (NR, PA, P, and A fragments, corresponding to residues 11–533, 534–1072, 647–865, and 870–1072, respectively) expressed in bacterial cells were purified as described elsewhere (Aizawa et al., 1991).

*Phosphorylation of MAP4 or Expressed Fragments by PKC.* MAP4 or bacterially expressed fragments were incubated with PKC in a reaction mixture consisting of 15 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 40 $\mu$M [$\gamma$-$^{32}$P]ATP (200–400 mCi/mmol), and activators of PKC (0.5 mM $CaCl_2$, 50 $\mu$g/mL PS, and 1 $\mu$g/mL OAG) (reaction mixture A) or in a reaction mixture consisting of 100 mM Pipes (pH 6.8), 0.1 mM [$\gamma$-$^{32}$P]ATP (100–200 mCi/mmol), 1 mM $MgCl_2$, 6% glycerol, and PKC activators (0.4 mM $CaCl_2$, 40 $\mu$g/mL PS, and 1 $\mu$g/mL OAG) (reaction mixture B). Reaction mixture A, which provides optimal conditions for the PKC reaction, was used only for the experiments to obtain $K_m$ and $V_{max}$ values for MAP4 phosphorylation. Reaction mixture B, in which the rate of MAP4 phosphorylation was slower than in reaction mixture A but in which the promotion of microtubule assembly by phosphorylated MAP4 was facilitated, was used for other experiments. After incubation at 30 °C, the reaction mixture was heat-treated at 100 °C for 4 min and centrifuged to remove denatured enzyme, leaving heat-stable MAP4 or MAP4 fragments in the supernatant. The amount of incorporated phosphate was determined as follows. After electrophoresis of the supernatants mentioned above, the gel was stained with Coomassie Brilliant Blue R-250 and dried. The MAP4 or MAP4 fragment bands were excised and their radioactivities were measured by a scintillation counter.

To measure the $K_m$ and $V_{max}$ values of PKC for the phosphorylation of MAP4, various concentrations of MAP4 were incubated with 1 unit/mL PKC [1 unit of the enzyme is defined as the activity to transfer 1 nmol of phosphate from ATP to peptide $MBP_{4-14}$ (Yasuda et al., 1990) per minute under optimal conditions (Kishimoto et al., 1983)]. Enzyme reactions were allowed to proceed at 30 °C for 6 min.

For the measurement of the maximal incorporation of phosphate into MAP4 or expressed fragments, 0.04–0.1 mg/mL protein was incubated with 5–10 units/mL PKC. Aliquots from the reaction mixtures were withdrawn every 15 min and the incorporated radioactivity was measured.

*Assay of Microtubule Assembly.* MAP4 was mixed with tubulin (1.5 mg/mL) at 0 °C in a reassembly buffer [RB: 100 mM Pipes (pH 6.8), 2 mM EGTA, and 1 mM $MgCl_2$] containing 0.5 mM GTP. Microtubule assembly was started by warming the solution at 37 °C. The time course of assembly was monitored by measuring the change in turbidity at 350 nm with a Gilford 260 spectrophotometer (Kotani et al., 1984).

*Chymotryptic Digestion.* Phosphorylated or unphosphorylated MAP4 (0.23 mg/mL) was digested with chymotrypsin (1.25 $\mu$g/mL) at 25 °C for the indicated times. The reaction was stopped by the addition of 100 mM PMSF in dimethylformamide to a final concentration of 2 mM.

*Cosedimentation of MAP4 Fragments with Microtubules.* Digested MAP4 was mixed with RB containing tubulin, taxol, and GTP at final concentrations of 1.0 mg/mL, 20 $\mu$M, and 0.5 mM, respectively. After incubation at 37 °C for 25 min, the microtubules and microtubule-binding fragments were collected as pellets by centrifugation at 100000*g* for 10 min at 4 °C, leaving fragments without affinity for microtubules in the supernatant.

*Determination of the Phosphorylated Site.* The P fragment (0.2 mg/mL) was phosphorylated by PKC (15 units/mL) in reaction mixture B (0.2 mL) containing 0.1 mM [$\gamma$-$^{32}$P]ATP (500 mCi/mmol) for 1 h, and the phosphorylated fragment was purified by high-performance liquid chromatography (HPLC) on a Vydac $C_{18}$ column with a linear gradient of acetonitrile from 0 to 70% (v/v) in 0.1% trifluoroacetic acid (TFA) for 40 min. Elution of the peptides was monitored by the measurement of absorbance at 210 nm. The purified fragment was dried and dissolved in 20 $\mu$L of 8 M urea and incubated for 30 min at room temperature. The solution was diluted with 60 $\mu$L of 0.1 M Tris-HCl (pH 9.0) containing 1/50 (w/w) LEP, and proteolysis was carried out at 37 °C for 18 h. The produced peptides were then separated by HPLC on a Vydac $C_{18}$ column with a linear gradient from 0 to 80% acetonitrile in 0.1% TFA for 100 min. Phosphopeptides were detected by counting the radioactivity of each fraction. The purified radioactive peptide was subjected to amino acid sequence analysis on a gas-phase sequencer [470A protein sequencer, Applied Biosystems, Inc. (Foster City, CA)].

*Determination of the Phosphorylated Serine Residue.* The purified phosphopeptide was lyophilized and treated with 25 $\mu$L of a solution consisting of ethanethiol, water, dimethyl sulfoxide, ethanol, and 5 N NaOH (9.9:33.1:33.1:13.2:10.7) at 50 °C for 1 h, as described by Meyer et al. (1986). Finally, 2.5 $\mu$L of acetic acid was added and the modified peptide was applied directly to the sequencer.

*Phosphoamino Acid Analysis.* The phosphopeptide fraction was dried in a glass tube and 0.3 mL of 6 N HCl was added. Hydrolysis was carried out by incubation at 110 °C for 2 h in vacuo. HCl was removed by two additions of 900 $\mu$L of water, with the mixture dried after each addition. The hydrolysate was dissolved in water containing unlabeled phosphoserine, phosphothreonine, and phosphotyrosine as internal markers. Thin-layer chromatography (cellulose MN 300-coated plates) was performed with a developing solution consisting of 1-butanol, isopropyl alcohol, formic acid, and water (3:1:1:1) (Swarup et al., 1981). The plate was dried and the positions of the markers and labeled amino acids were visualized by ninhydrin reaction and autoradiography, respectively.

*Other Methods.* Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) with the following protein markers: myosin heavy chain (200 kDa), phosphorylase *b* (98 kDa), bovine

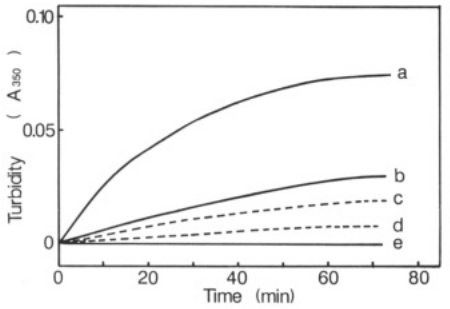


FIGURE 1: Effect of phosphorylation of MAP4 by PKC on its ability to promote microtubule assembly. In order to prepare phosphorylated and unphosphorylated MAP4, MAP4 (0.24 mg/mL) was incubated with PKC (13 units/mL) in reaction mixture B (0.3 mL) with or without 0.1 mM ATP as described under Materials and Methods. In the case of unphosphorylated MAP4, 0.1 mM ATP was added to the reaction mixture after termination of the reaction by heat treatment. Samples were centrifuged at 100000*g* for 12 min to remove denatured enzyme. Phosphorylated or unphosphorylated MAP4 was mixed with tubulin (1.5 mg/mL) and GTP (0.5 mM), and the time course of microtubule assembly was monitored by measuring the turbidity at 350 nm. (a, c) 0.19 mg/mL unphosphorylated and phosphorylated MAP4, respectively. (b, d) 0.095 mg/mL unphosphorylated and phosphorylated MAP4, respectively. (e) without MAP4. In this experiment, 0.56 mol of phosphate was incorporated/mol of MAP4.

serum albumin (68 kDa), tubulin (55 kDa), actin (42 kDa), glyceraldehyde-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21 kDa).

## RESULTS

*In Vitro Phosphorylation of MAP4 by PKC.* To test whether MAP4 can be a substrate for PKC, we incubated MAP4 with the kinase in the presence or absence of PS, OAG, and $Ca^{2+}$. MAP4 was phosphorylated only in the presence of activators of PKC (data not shown). Maximum phosphate incorporation was 0.85 mol/mol of MAP4. The apparent $K_m$ for MAP4 was calculated to be 2 $\mu$M from a Lineweaver–Burk plot (data not shown). This value is lower than those for $MBP_{4-14}$ (7 $\mu$M) and H1 histone (3–10 $\mu$M) (Yasuda et al., 1990). When 0.04 unit of PKC was used, the $V_{max}$ for MAP4 phosphorylation was 3.4 pmol/min. This value is one-twelfth that for peptide $MBP_{4-14}$. Since the $V_{max}$ for H1 histone was reported to be two-fifths that for $MBP_{4-14}$ (Yasuda et al., 1990), the $V_{max}$ for MAP4 was estimated to be one-fifth that for H1 histone. Judging from the $K_m$ and $V_{max}$ values for MAP4, it is apparent that MP4 is a good substrate for PKC.

*Assembly Promotion Activity of Phosphorylated MAP4.* Next, we investigated the effect of phosphorylation on the ability of MAP4 to promote microtubule assembly. Phosphorylated or unphosphorylated MAP4 was mixed with tubulin and incubated at 37 °C. When phosphorylated MAP4 (0.56 mol of phosphate/mol of MAP4) was used, the initial rate of tubulin polymerization was reduced to about one-fourth that with unphosphorylated MAP4 (Figure 1).

*Identification of the Phosphorylated Region of MAP4.* Since the phosphorylation of MAP4 by PKC remarkably reduced its assembly-promoting activity, we attempted to identify the phosphorylated region using MAP4 fragments expressed in *Escherichia coli*. When the N-terminal half fragment (NR fragment) or the C-terminal half fragment (PA fragment) (Figure 2) was treated with PKC in the presence of activators, phosphate was incorporated into the PA fragment (Figure 3A, b, lanes 3 and 4) with a maximal phosphorylation of 0.85 mol/mol of fragment. On the other hand, no phosphorylation was observed in the NR fragment (Figure 3A, b, lanes 1 and 2). In order to narrow down the site of the phosphorylated

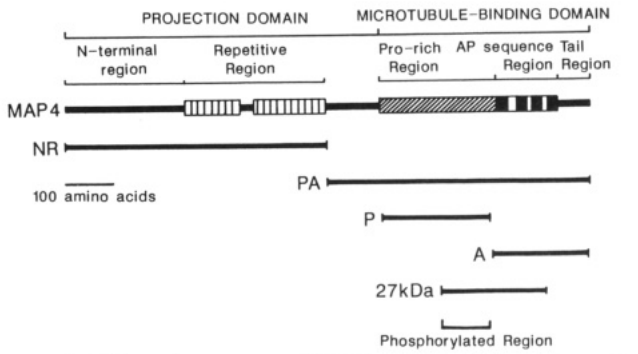


FIGURE 2: Schematic structure of MAP4, expressed fragments, and the 27-kDa fragment. The regions of MAP4 corresponding to the fragments are indicated by thick horizontal lines. The phosphorylated region, deduced from the data in Figures 3 and 4, is indicated by a bracket.

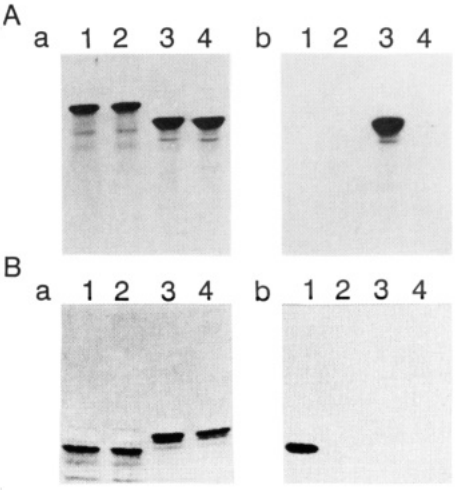


FIGURE 3: Phosphorylation of expressed fragments of MAP4 by PKC. (A) NR (lanes 1 and 2) or PA (lanes 3 and 4) fragments (0.15 mg/mL each) were incubated for 1 h with PKC (3 units/mL) in reaction mixture B with (lanes 1 and 3) or without (lanes 2 and 4) PS, OAG, and $Ca^{2+}$. The reaction was terminated by heat treatment. After centrifugation, the supernatants were electrophoresed. (a) Coomassie Brilliant Blue staining. (b) Autoradiography. (B) P (lanes 1 and 2) or A (lanes 3 and 4) fragment (0.22 mg/mL each) was mixed with PKC (3 units/mL) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of PS, OAG, and $Ca^{2+}$. After the reaction was stopped by heat treatment, the samples were centrifuged and the supernatants were electrophoresed. (a) Coomassie B·illiant Blue staining. (b) Autoradiography.

region, we measured the ability of the P fragment and the A fragment (Figure 2) to be phosphorylated by PKC. It was clearly shown that the P fragment was phosphorylated by PKC (Figure 3B, b, lanes 1 and 2) while the A fragment was not (Figure 3B, b, lanes 3 and 4). Maximum incorporation of phosphate into the P fragment was 0.60 mol/mol of fragment. This value is not so different from that in the case of MAP4 (0.85 mol/mol) and the PA fragment (0.85 mol/mol).

Next we investigated the phosphorylated region in intact MAP4 pretreated with PKC by analyzing the radioactivity of chymotryptic fragments. When fragments produced by digestion of unphosphorylated MAP4 with chymotrypsin for various lengths of time were incubated with taxol-stabilized microtubules and centrifuged, microtubule-binding fragments precipitated with the microtubules (Figure 4, P) leaving fragments without binding activity in the supernatant (Figure 4, S). Several intermediate fragments and a chymotrypsin-resistant stable fragment with a molecular mass of 27 kDa were shown to have the ability to bind to microtubules (Figure 4, P). When chymotryptic fragments were incubated without

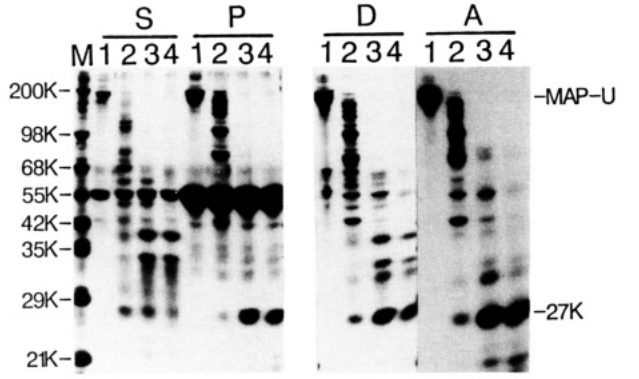


FIGURE 4: Chymotryptic digestion of unphosphorylated and phosphorylated MAP4. Unphosphorylated MAP4 (0.23 mg/mL) was digested with chymotrypsin (1.25 μg/mL) for 0 min (lane 1), 2 min (lane 2), 15 min (lane 3), or 30 min (lane 4). Microtubule-binding fragments (lanes labeled P) were separated from fragments without affinity for microtubules (lanes labeled S) by cosedimentation with taxol-stabilized microtubules. Aliquots of 20 μL from the supernatants and precipitates were electrophoresed on a 7.5–15% (w/v) polyacrylamide gradient gel. Phosphorylated MAP4 was prepared by incubating MAP4 (0.23 mg/mL) with PKC (1.2 units/mL) for 1 h and digesting as described above. Aliquots of 20 μL were electrophoresed. The gel was stained (lanes labeled S, P, and D) and autoradiographed (lanes labeled A). M, markers. Molecular masses are indicated on the left.

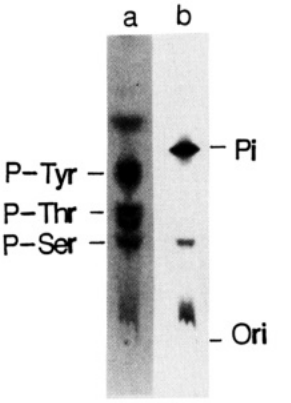


FIGURE 5: Phosphoamino acid analysis. The phosphopeptide purified by HPLC was hydrolyzed and the amino acids were separated by thin-layer chromatography with phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) as internal markers. The plate was subjected to ninhydrin reaction (a), and the radioactive phosphoamino acids were detected by autoradiography (b). Visualization of the radioactive bands was performed with an image analyzer, BAS 2000 (Fujix, Japan).

microtubules, no precipitation of the fragments was observed. We have already reported that the 27-kDa fragment derives from the microtubule-binding domain of MAP4 as judged from the experimental result that the purified 27-kDa fragment stimulates the polymerization of tubulin into morphologically normal microtubules and that the fragment cosediments with the formed microtubules (Aizawa et al., 1987). In parallel with the above experiment, phosphorylated MAP4 was digested with chymotrypsin for the same lengths of time and the fragments produced were analyzed by electrophoresis (Figure 4, D) and autoradiography (Figure 4, A). The digestion pattern of phosphorylated MAP4 was almost identical with that of unphosphorylated MAP4. Among the phosphopeptides, the most prominent was the 27-kDa fragment (Figure 4, D and A, lanes 3 and 4). After digestion for 15 min, about 70% of the radioactivity in MAP4 was recovered in the 27-kDa fragment. It was strongly suggested that the phosphorylation of intact MAP4 occurred almost exclusively in the region corresponding to the 27-kDa fragment. Using a gas-phase amino acid sequencer, we found the sequence of the amino terminus of the 27-kDa fragment to be ASPGSTSRNL, which corresponds to $Ala^{764}$–$Leu^{773}$ of MAP4. Judging from the cleavage specificity of chymotrypsin and the molecular mass of the 27-kDa fragment, the carboxyl terminus of the 27-kDa fragment is thought to be $Phe^{978}$. Therefore, it is concluded that the 27-kDa fragment corresponds to $Ala^{764}$–$Phe^{978}$, consisting of the C-terminal half of the Pro-rich region and three repeats of the AP sequence (Figure 2). This conclusion is supported by the fact that the amino acid composition calculated for the sequence $Ala^{764}$–$Phe^{978}$ (data not shown) is consistent with the reported data on the 27-kDa fragment (Aizawa et al., 1987). Judging from the data obtained from the phosphorylation of the expressed fragments and analysis of the chymotryptic fragments derived from phosphorylated MAP4, the phosphorylated site of MAP4 is located within the sequence of residues 764–865 (Figure 2).

*Determination of the Phosphorylated Site of MAP4.* We used the P fragment to identify the phosphorylated site of MAP4. The P fragment was phosphorylated by PKC and subjected to digestion by LEP. The resultant fragments were fractionated by reverse-phase HPLC with the $A_{210}$ of the eluate monitored and the radioactivity of each peak measured. It was found that the major part of the radioactivity was recovered in a single peak (data not shown). This peak was not detected when unphosphorylated P fragment was digested by LEP and subjected to HPLC in the same way. It is considered that phosphorylation causes a change in the charge characteristics of a peptide and hence changes the chromatographic behavior of the peptide to produce a novel peak.

Phosphoamino acid analysis of the phosphopeptide fraction indicated that phosphorylation occurred on a Ser residue (Figure 5). Amino-terminal amino acid sequence analysis revealed that the sequence of the phosphopeptide was STTTSSVK. This sequence corresponds to $Ser^{810}$–$Lys^{817}$ of MAP4. Since this phosphopeptide contains three serine residues, the exact phosphorylation site had to be defined. For this purpose, the fragment was treated with ethanethiol under alkaline conditions to specifically convert the phosphoserine residue to *S*-ethylcysteine (Meyer et al., 1986). After modification, the position of the *S*-ethylcysteine residue was identified by gas-phase sequencing. A release of *S*-ethylcysteine was observed on the sixth cycle. We conclude that $Ser^{815}$ is the site of MAP4 phosphorylated by PKC.

## Discussion

In this study, we found that PKC catalyzes the phosphorylation of MAP4 at a specific Ser residue ($Ser^{815}$) and that phosphorylation suppresses the ability of MAP4 to stimulate microtubule assembly in vitro.

It is intriguing that the phosphorylation occurs in the Pro-rich region, a region recently revealed to support the binding of the AP sequence region to microtubules (Aizawa et al., 1991). It has been suggested that an electrostatic interaction between a basic region in MAP and an acidic region in tubulin is important for the association of the two proteins (Erickson et al., 1976). Thus, phosphorylation may suppress the activity of the basic Pro-rich region and, consequently, regulate the assembly-promoting activity of MAP4. However, phosphorylation of only one amino acid residue in the Pro-rich region, which contains many basic amino acid residues, does not appear to alter the net positive charge significantly. One possible explanation for the effect of this single phosphorylation is that $Ser^{815}$ is directly involved in the interaction of MAP4 with tubulin. It is also possible that the phosphorylation of the $Ser^{815}$ residue alters the three-dimensional structure of the

microtubule-binding domain of MAP4 and causes the reduction in its activity.

Other MAPs, such as MAP2 and $\tau$, are also known to be phosphorylated by PKC to reduce their activity to promote microtubule assembly (Hoshi et al., 1987, 1988). The microtubule-binding domains of these MAPs also contain Pro-rich regions that resemble that of MAP4 (Aizawa et al., 1990). It is possible that phosphorylation of the Pro-rich regions of MAP2 and $\tau$ may also regulate their ability to promote tubulin polymerization.

When we examined the phosphorylation of MAP4 by cyclic AMP dependent protein kinase or casein kinase II, the efficiency of phosphate incorporation was low. Moreover, an experiment with the expressed fragments of MAP4 revealed that the site(s) phosphorylated by these kinases exists in the projection domain. These results suggest that phosphorylation by these kinases does not affect the assembly-promoting activity of MAP4.

It is well-known that the interphase microtubule network disappears at the end of the $G_2$ phase and that the mitotic apparatus, another type of microtubular architecture, is constructed in M phase. It has been reported that MAPs regulate the formation and stability of microtubules in vivo (Drubin & Kirschner, 1986; Kanai et al., 1989; Lewis et al., 1989). On the other hand, it has been shown that a PKC homologue of *Saccharomyces cerevisiae* is necessary at a certain point of the $G_2$ phase in the cell division cycle (Levin et al., 1990). This suggests that PKC may play an important role in the $G_2$–M phase transition in mammalian cells, too. Thus, the phosphorylation of MAP4 by PKC might be involved in the destruction of the interphase microtubule network at the end of $G_2$. In the course of this study, we found that *cdc2* kinase, an M-phase-specific protein kinase, also phosphorylates the Pro-rich region of MAP4 and, consequently, suppresses the assembly-promoting activity of MAP4.[2] This region contains putative phosphorylation sites preferred by *cdc2* kinase, which are different from that preferred by PKC. Taking all these data into consideration, it seems possible that several protein kinases (including PKC and *cdc2* kinase) synergistically regulate the activity of MAP4 through phosphorylation of the Pro-rich region, a region that may function as a regulatory region for the activity of MAP4.

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## References

Aizawa, H., Murofushi, H., Kotani, S., Hisanaga, S., Hirokawa, N., & Sakai, H. (1987) *J. Biol. Chem. 262*, 3782–3787.

Aizawa, H., Kawasaki, H., Murofushi, H., Kotani, S., Suzuki, K., & Sakai, H. (1989) *J. Biol. Chem. 264*, 5885–5890.

Aizawa, H., Emori, Y., Murofushi, H., Kawasaki, H., Sakai, H., & Suzuki, K. (1990) *J. Biol. Chem. 265*, 13849–13855.

Aizawa, H., Emori, Y., Mori, A., Murofushi, H., Sakai, H., & Suzuki, K. (1991) *J. Biol. Chem. 266*, 9841–9846.

Binder, L. I., Frankfurter, A., & Rebhun, L. I. (1985) *J. Cell Biol. 101*, 1371–1378.

Bulinski, J. C., & Borisy, G. G. (1980) *J. Biol. Chem. 255*, 11570–11576.

Burns, R. G., Islam, K., & Chapman, R. (1984) *Eur. J. Biochem. 141*, 609–615.

Cleveland, D. W., Hwo, S. Y., & Kirschner, M. W. (1977) *J. Mol. Biol. 116*, 207–225.

Drubin, D. G., & Kirschner, M. W. (1986) *J. Cell Biol. 103*, 2739–2746.

Dustin, P. (1984) *Microtubules*, 2nd ed., Springer-Verlag, Heidelberg, Germany.

Erickson, H. P., & Voter, W. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2813–2817.

Goedert, M., Wischik, C. M., Crowther, R. A., Walker, J. E., & Klug, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4051–4055.

Himmler, A., Drechsel, D., Kirschner, M., & Martin, D. W. (1989) *Mol. Cell. Biol. 9*, 1381–1388.

Hoshi, M., Nishida, E., Miyata, Y., Sakai, H., Miyoshi, T., Ogawara, H., & Akiyama, T. (1987) *FEBS Lett. 217*, 237–241.

Hoshi, M., Akiyama, T., Shinohara, Y., Miyata, Y., Ogawara, H., Nishida, E., & Sakai, H. (1988) *Eur. J. Biochem. 174*, 225–230.

Huber, G., & Matus, A. (1984) *J. Cell. Biol. 98*, 777–781.

Kanai, Y., Takemura, R., Oshima, T., Mori, H., Ihara, Y., Yanagisawa, M., Masaki, T., & Hirokawa, N. (1989) *J. Cell Biol. 109*, 1173–1184.

Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol. 80*, 266–276.

Kishimoto, A., Kajikawa, N., Shiota, M., & Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 1156–1164.

Kitano, T., Go, M., Kikkawa, U., & Nishizuka, Y. (1986) *Methods in Enzymology* (Conn, P. M., Ed.) pp 349–352, Academic Press, New York.

Kotani, S., Murofushi, H., Nishida, E., & Sakai, H. (1984) *J. Biochem. (Tokyo) 96*, 959–969.

Kotani, S., Murofushi, H., Maekawa, S., Aizawa, H., & Sakai, H. (1988) *J. Biol. Chem. 263*, 5385–5389.

Kuznezov, S. A., Rodionov, V. I., Gelfand, V. I., & Rosenblat, V. A. (1981) *FEBS Lett. 135*, 237–240.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lee, G., Cowan, N., & Kirschner, M. (1988) *Science 239*, 285–288.

Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., & Thorner, J. (1990) *Cell 62*, 213–224.

Lewis, S. A., Villasante, A., Sherline, P., & Cowan, N. J. (1986) *J. Cell Biol. 102*, 2098–2105.

Lewis, S. A., Wang, D., & Cowan, N. J. (1988) *Science 242*, 936–939.

Lewis, S. A., Ivanov, I. E., Lee, G. H., & Cowan, N. J. (1989) *Nature 342*, 498–505.

Lindwell, G., & Cole, R. D., (1984) *J. Biol. Chem. 259*, 5301–5305.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Meyer, H. E., Hoffmann-Posorske, E., Korte, H., & Heilmeyer, L. M. G., Jr. (1986) *FEBS Lett. 204*, 61–66.

Murofushi, H., Kotani, S., Aizawa, H., Hisanaga, S., Hirokawa, N., & Sakai, H. (1986) *J. Cell Biol. 103*, 1911–1919.

Murphy, D. B., & Borisy, G. G. (1975) *Proc. Acad. Natl. Sci. U.S.A. 72*, 2696–2700.

Parysek, L. M., Asnes, C. F., & Olmsted, J. B. (1984) *J. Cell Biol. 99*, 1309–1315.

Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.

Swarup, G., Cohen, S., & Garbers, D. L. (1981) *J. Biol. Chem. 256*, 8197–8201.

[2] H. Aizawa, M. Kamijo, A. Mori, H. Murofushi, and H. Yasuda, unpublished data.

Vallee, R. B., Bloom, G. S., & Luca, F. C. (1984) *Molecular Biology of the Cytoskeleton* (Borisy, G. G., Cleveland, D. W., & Murphy, D. B., Eds.) pp 111-130, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Weatherbee, J. A., Luftig, R. B., & Weihing, R. R. (1980) *Biochemistry 19*, 4116-4123.

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858-1862.

Yasuda, I., Kishimoto, A., Tanaka, S., Tominaga, M., Sakurai, A., & Nishizuka, Y. (1990) *Biochem. Biophys. Res. Commun. 166*, 1220-1227.

# Influx of Extracellular Calcium Is Required for the Membrane Translocation of 5-Lipoxygenase and Leukotriene Synthesis

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ABSTRACT: Our studies assessed the effects of increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) on leukotriene synthesis and membrane translocation of 5-lipoxygenase (5LO). The calcium ionophore ionomycin and the tumor promoter thapsigargin stimulated leukotriene production and translocation of 5-lipoxygenase to the membrane. Both agents elicited prolonged rises in $[Ca^{2+}]_i$. Leukotriene $C_4$ production associated with $[Ca^{2+}]_i$ in cells stimulated with various concentrations of ionomycin and thapsigargin suggests that a threshold $[Ca^{2+}]_i$ level of approximately 300-400 nM is required. In the absence of extracellular $Ca^{2+}$, both the ionomycin- and thapsigargin-induced rises in $[Ca^{2+}]_i$ were transient, indicating that the prolonged $[Ca^{2+}]_i$ elevation is due to an influx of extracellular $Ca^{2+}$. Addition of EGTA to the external medium before, or at different times during, the treatment with ionomycin or thapsigargin instantaneously inhibited 5LO translocation and leukotriene synthesis, indicating that $Ca^{2+}$ influx plays an essential role in 5LO membrane translocation and leukotriene synthesis. No leukotriene production was detected when cells were stimulated by a physiological stimulus of leukotriene $D_4$. The addition of 100 nM leukotriene $D_4$ triggered peak rises in $[Ca^{2+}]_i$ that were comparable to those achieved by the ionomycin and thapsigargin. However, the leukotriene $D_4$ induced rise was transient and rapidly declined to a lower but still elevated steady-state level, which was attributed to $Ca^{2+}$ influx. Stimulation with 100 nM leukotriene $D_4$ for 15 s increased the cellular levels of 1,4,5-inositol triphosphate ($IP_3$), 1,3,4-$IP_3$, and 1,3,4,5-inositol tetraphosphate ($IP_4$). In contrast, 100 nM thapsigargin had no effect on generating inositol phosphate after 15, 60, or 300 s of treatment. These results argue against an essential role for inositol phosphates in leukotriene synthesis and indicate that the stimulation of 5-lipoxygenase membrane translocation and leukotriene synthesis is a consequence of a sustained increase in $[Ca^{2+}]_i$ resulting from an influx of external $Ca^{2+}$.

Studies with a variety of cell systems have led to the view that a rise in $[Ca^{2+}]_i$[1] is an obligatory step in the synthesis of leukotrienes. The main evidence for this conclusion is summarized as follows: First, leukotriene (LT) synthesis can be obtained after binding of agonists to cell surface receptors, which results in an increase of $[Ca^{2+}]_i$. For example, macrophages synthesize $LTC_4$ in response to several $Ca^{2+}$-mobilizing stimuli such as opsonized zymosan (Humes et al., 1982; Tripp et al., 1985) and immunoglobulin E complexed with antigen (Rouzer et al., 1982). Second, calcium ionophore A23187 induces leukotriene synthesis (Borgeat et al., 1979), presumably through a rise in $[Ca^{2+}]_i$. Third, at least two enzymes in the leukotriene synthesis pathway require $Ca^{2+}$: phospholipase $A_2$, for release of arachidonic acid (Wijkander & Sundler, 1989; Leslie et al., 1988), and 5-lipoxygenase (5LO), for synthesis of 5-hydroperoxyeicosatetraenoic acid and $LTA_4$ (Jakschik & Lee, 1980). In addition, $Ca^{2+}$ regulates the membrane translocation of 5LO (Rouzer & Kargman, 1988; Wong et al., 1988). It has been hypothesized that a rise in $[Ca^{2+}]_i$ induces the binding of 5LO to a specific integral membrane protein, which might aid the enzyme in obtaining substrate released from membrane phospholipid stores (Rouzer et al., 1990; Dixon et al., 1990).

Despite these findings, some observations have cast doubt on the simplistic view that a rise in $[Ca^{2+}]_i$ is necessary or sufficient to trigger the synthesis of leukotrienes. Recent reports have demonstrated that some stimuli induce a rise in $[Ca^{2+}]_i$ that is insufficient for generating leukotrienes; for example, human leukocytes stimulated with [(*N*-formylmethionyl)leucinyl]phenylalanine or complement $C_{5a}$ (Clancy et al., 1983; Haines et al., 1987) fail to generate a significant amount of leukotrienes. In order to understand the precise

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[1] Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium; AM, acetoxymethyl ester; $IP_3$, inositol triphosphate; $IP_4$, inositol tetraphosphate; LT, leukotriene; 5HETE, 5-hydroxytetraeicosanoic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH buffer, Krebs-Ringer-Henseleit buffer; 5LO, 5-lipoxygenase; RBL, rat basophilic leukemia; Tg, thapsigargin.

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