Importance of Protein Kinase C Targeting for the Phosphorylation of Its Substrate, Myristoylated Alanine-rich C-kinase Substrate*

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We visualized the translocation of myristoylated alanine-rich protein kinase C substrate (MARCKS) in living Chinese hamster ovary-K1 cells using MARCKS tagged to green fluorescent protein (MARCKS-GFP). MARCKS-GFP was rapidly translocated from the plasma membrane to the cytoplasm after the treatment with phorbol ester, which translocates protein kinase C (PKC) to the plasma membrane. In contrast, PKC activation by hydrogen peroxide, which was not accompanied by PKC translocation, did not alter the intracellular localization of MARCKS-GFP. Non-myristoylated mutant of MARCKS-GFP was phosphorylated in cells treated with hydrogen peroxide, whereas non-myristoylated mutant of MARCKS-GFP was phosphorylated in cells treated with hydrogen peroxide but not with phorbol ester. Phosphorylation of wild-type MARCKS-GFP was observed in cells treated with phorbol ester but not with hydrogen peroxide, whereas non-myristoylated mutant of MARCKS-GFP was phosphorylated in cells treated with hydrogen peroxide but not with phorbol ester. Phosphorylation of both MARCKS-GFPs reduced the amount of F-actin. These findings revealed that PKC targeting to the plasma membrane is required for the phosphorylation of membrane-associated MARCKS and that a mutant MARCKS existing in the cytoplasm can be phosphorylated by PKC activated in the cytoplasm without translocation but not by PKC targeted to the membrane.

Protein kinase C (PKC)

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is a family of related serine/threonine kinases and plays a key role in cellular responses such as neurotransmission, gene expression, and cell growth and differentiation (1, 2). This multifunctional enzyme consists of at least 10 subtypes that can be classified into three groups based on structure, cPKC (α, βI, βII, and γ), nPKC (δ, ε, η, and θ), and aPKC (ζ and λ) (3, 4). Each subtype has shown different enzymological properties and distinct cellular distribution, suggesting specific functions of each PKC subtype (5–8). The individual function of each subtype has been studied by determining subtype-specific activation processes or subtype-specific substrates in the downstream of the PKC pathway (3, 9–11), but the roles of individual subtypes have not been fully clarified yet. Studying the electron microscopic co-localization of PKC subtypes and substrate has been thought to be an attractive way to learn about the functional specificity of PKC subtypes, but it was also demonstrated that PKC translocates to different subcellular compartments after activation by physiological stimuli. The mechanism of PKC translocation was then studied in living cells using green fluorescent protein (GFP)-tagged PKC, and it has been shown that each PKC subtype has a spatially and temporally different targeting mechanism that depends on the extracellular signals, contributing to the sub-specific functions of PKC (12–14).

Myristoylated alanine-rich protein kinase C substrate (MARCKS) is a major PKC substrate that is distributed in various cell types (15–23). MARCKS has been implicated in cell motility, phagocytosis, membrane traffic and mitogenesis (16, 24–30). MARCKS has three highly conserved regions, a myristoylation site at the N terminus, a highly conserved domain of unknown function (MARCKS homology 2 domain), and a basic effector domain that contains multiple PKC phosphorylation sites (31–34). MARCKS is localized on the plasma membrane, and the binding of MARCKS to the plasma membrane requires hydrophobic insertion of its myristate chain into the bilayer and also electrostatic interaction of the cluster of the basic residues in the effector domain with acidic lipids (35–38). Mutation of N-terminal glycine results in a non-myristoylated form of MARCKS being localized in the cytosol (39). Phosphorylation by PKC induces the translocation of MARCKS from membrane fraction to cytosolic fraction by introducing negative charges into the basic cluster (27, 36, 40–42). The binding of Ca2+ -calmodulin to the basic effector domain also induces translocation from the membrane (41). Glaser et al. (43) reported that phosphorylation of MARCKS by PKC allows PLC to produce a burst of inositol 1,4,5-trisphosphate and diacylglycerol, but the physiological significance of phosphorylation-dependent translocation of MARCKS has not been elucidated.

In the present study, to clarify the physiological significance of PKC targeting and to elucidate the importance of PKC targeting in the phosphorylation of a substrate by the enzyme, the movement of MARCKS was monitored in living cells using GFP-tagged MARCKS. Also, the phosphorylation of MARCKS and of its mutants was examined after PKC activation with or without PKC translocation.

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1 The abbreviations used are: PKC, protein kinase C; CHO, Chinese hamster ovary; GFP, green fluorescent protein; MARCKS, myristoylated alanine-rich C-kinase substrate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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Fig. 1. Constructs of MARCKS-GFP fusion protein and its mutants. A, BS555. Wild-type MARCKS is a rod-shaped molecule with at least three functional domains. It is myristoylated at its N terminus, and it also has an MARCKS homology 2 domain (MH2) and a basic effector domain (ED). The basic domain containing the phosphorylation sites binds calmodulin and actin. MARCKS and GFP were bound at the C terminus of MARCKS, B, BS644. The N-terminal glycine has been replaced with alanine, resulting in a non-myristoylated molecule. C, BS580. The serine residues of PKC phosphorylation sites are mutated to alanine.

EXPERIMENTAL PROCEDURES

Materials—TPA and UTP were purchased from Sigma. Hydrogen peroxide was from Santoku Chemical Industries Co. (Tokyo, Japan). Staurosporine was from Kyowa Pure Pharmaceutical Industries (Osaka, Japan). All other chemicals used were of analytical grade.

Cell Culture—Strain CHO-K1 (ATCC CCL 61) was purchased from the American Type Culture Collection. CHO-K1 cells were cultured in Ham’s F-12 medium supplemented with 44 mM NaHCO₃ and 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂. The medium was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), and the fetal bovine serum was not heat-inactivated.

Construct of Plasmids Encoding MARCKS-GFP Fusion Protein—An expression plasmid for mammalian cells (designated as BS554) that can fuse humanized GFP (a kind gift from Dr. Umezono, Kyoto University) at the C terminus of the target protein was prepared as described previously (14). cDNA for MARCKS/80K (designated as BS 518). A MARCKS cDNA with EcoRI site at both the 5′ and 3′ termini was produced by PCR using BS 518 as a template. The sense and antisense primers used were 5′-TTGAATTCAGCATGGCTGCCCAG-3′ and 5′-TCCGGGATCTCCTGTGCCCC-3′, respectively. After digestion with EcoRI, the PCR products were subcloned into EcoRI site in BS354. Site-directed mutagenesis was performed using an Exsiste PCR site-directed mutagenesis kit (Stratagene, La Jolla, CA) with BS 518 as a template, in order to make a mutant MARCKS/GFP, the three putative PKC phosphorylation sites of which (Ser-152, Ser-156, and Ser-163) were substituted with Ala (designated as BS 580) (Fig. 1B). For mutant BS 580, the PCR primers used were 5′-CCCTGAGGTGCAGCCTGCCCCAG-3′ and 5′-CCCTGAGGTGCAGCCTGCCCCAG-3′. To add, mutagenic MARCKS-GFP was not myristoylated and was unable to bind plasma membrane, a glycine residue at position 2 was replaced with an alanine residue. cDNAs and BS644 were transfected to 10⁶ CHO-K1 cells by lipofection using TransIT™-LT2 (Mirus, Madison, WI) according to the manufacturer’s standard protocol. The fluorescence of homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After the sonication (UD-210 Tomy Seiko, Tokyo, Japan) (output, 5; duty, 50%; 10 times at 4 °C), samples were centrifuged at 400,000 × g for 30 min at 4 °C (Hitachi, Tokyo, Japan), and supernatant was collected as the cytosol fraction. The pellets were sonicated with 500 μl of homogenate buffer containing 1% Triton X-100 and centrifuged at 19,000 × g for 15 min, and the supernatant was collected as the particulate fraction. For immunoblotting, the same amount of samples from each fraction (20 μg) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophotically transferred onto polyvinylidene difluoride filters (Millipore, Bedford, MA). Nonspecific binding sites on the polyvinylidene difluoride filters were blocked by incubation with 2% bovine serum albumin in 0.01 M PBS containing 0.03% Triton X-100 for 1 h. The polyvinylidene difluoride filters were then incubated with the anti-phospho-MARCKS polyclonal antibody (anti-pp-3) (diluted 1:200) or the anti-GFP polyclonal antibody (CLONTECH, Palo Alto, CA) (diluted 1:1000) for 1 h at 25 °C. After washing with 0.01 M PBS containing 0.03% Triton X-100, the filters were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (The Jackson Laboratory, Arlingtontown, HI) (diluted 1:10000) for 30 min. After three rinses, the immunoreactive bands were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

For immunoprecipitation of MARCKS-GFP or its mutants, transfected cells were harvested with 0.5 ml of homogenate buffer containing 1% Triton X-100 and homogenized by sonication. After centrifugation at 19,000 × g for 15 min at 4 °C, the supernatant was rotated with an anti-GFP polyclonal antibody (diluted 1:50) (Molecular Probes, Eugene, OR) for 2 h at 4 °C and then with protein A-Sepharose for an additional 2 h. Samples were centrifuged at 2,000 × g for 5 min at 4 °C, and pellets were washed three times with phosphate-buffered saline containing 0.1 M NaCl and 0.05% Tween 20. Finally, the pellet was suspended in 50 μl of PBS(−) and used for phosphorylation or immunoblotting studies as described below.

Observation of MARCKS-GFP Translocation—CHO-K1 cells expressing MARCKS/GFP or its mutants were spread onto glass-bottomed culture dishes (MatTek, Ashland, MA) and cultured for at least 16 h before observation. The culture medium was replaced with normal Hepes buffer composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes, and 10 mM glucose, pH 7.3. The fluorescence of GFP was monitored under a confocal laser scanning fluorescence microscope (Carl Zeiss, Jena, Germany) at 488-nm argon excitation with a 515-nm-long pass barrier filter. Translocation of the fusion protein was triggered by the addition of various stimulators at high concentrations into the Hepes buffer to obtain the appropriate final concentrations. All experiments were performed at 37 °C.

Immunostaining of CHO-K1 Cells Expressing MARCKS and Its Fusion Protein by Anti-phospho-MARCKS Antibody—CHO-K1 cells cultured in glass-bottomed dishes expressing MARCKS and its fusion protein were observed under a confocal laser scanning fluorescence microscope. After the conformation of their translocation by TPA, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS for 30 min. After three washes with 0.1 M PBS, the cells were treated with PBS containing 0.3% Triton X-100 and 10% normal goat serum for 20 min. Cells were sequentially incubated with anti-phospho-MARCKS polyclonal antibody (pp-3, diluted 1:1000) for 40 min in PBS with 0.03% Triton X-100 (PBS-T) and 10% normal goat serum and then with Cy3-labeled goat anti-rabbit IgG for 30 min at room temperature. The fluorescence of phosphate-MARCKS-like immunoreactivity was observed.
under a confocal laser scanning fluorescent microscope at 588-nm argon excitation with a 590-nm-long pass barrier filter. GFP fluorescence was observed at 488-nm argon excitation with a 515-nm-long pass barrier filter.

**Kinase Assay of Endogenous δ-PKC and of Recombinant δ-PKC-GFP**—Endogenous δ-PKC was immunoprecipitated from normal CHO-K1 cells using anti-δ-PKC antibody, and δ-PKC-GFP was immunoprecipitated from CHO-K1 cells transfected with δ-PKC-GFP. The immunoprecipitated samples (10 μl of suspended pellet) were used for kinase assays. The kinase activity was assayed by measuring the incorporation of [32P] into calf thymus H1 histone from [γ-32P]ATP without any activators, such as phosphatidylserine, diolein, and Ca2+, as described previously (47).

**Phosphorylation of MARCKS and Its Mutant by δ-PKC in Vitro**—MARCKS-GFP and its mutants were prepared by immunoprecipitation using anti-GFP antibody from CHO-K1 cells transfected with the corresponding cDNA. The standard phosphorylation assay was performed in the reaction mixture containing MARCKS-GFP or its mutants, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 20 μM ATP, 16 μg/ml phosphatidylserine, 1.6 μg/ml diolein, 15–50 kBq of [γ-32P]ATP, and the immunoprecipitated PKCδ using monoclonal anti-δ-PKC antibody (Transduction Laboratories, Lexington, KY) in a total volume of 15 μl.

The reaction was carried out for 10 min at 30 °C. The reaction was stopped by the addition of 15 μl of 2X SDS-sample buffer, and the phosphorylated proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis.

**Fluorescence Photobleaching**—For photobleaching experiments, a squared region within a plane of the cell was photobleached by scanning for 30 s with the highest laser power. Recovery of fluorescence into the photobleached region was then observed by imaging the entire cell by confocal fluorescent microscopy with low laser power at the indicated times after photobleaching. In all of the images, the noise levels were reduced by line scan averaging.

**Actin Staining of CHO-K1 Cells Expressing MARCKS-GFP and Its Mutants**—The CHO-K1 cells expressing wild-type and mutant MARCKS-GFP were cultured in glass-bottomed dishes. The cells were treated with TPA and H2O2 in the KRH buffer and then fixed with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.01 M PBS (pH 7.4) for 30 min. After washing twice with PBS, the cells were treated with PBS containing 0.3% Triton X-100 and 5% normal goat serum for 10 min. For the staining of F-actin, cells were incubated with rhodamine-phalloidin (0.18–0.36 units/ml) for 40 min in PBS-T and 5% normal goat serum. After three washes with PBS-T for 10 min, the fluorescence of stained F-actin was observed by imaging the entire cell by confocal laser scanning fluorescent microscope at 588-nm argon excitation with a 590-nm-long pass barrier filter.

**RESULTS**

**Immunoblot Analysis of MARCKS-GFP Fusion Protein**—We examined the effects of GFP fusion on the characteristics of MARCKS by immunoblotting. As shown in Fig. 2, MARCKS-GFP was predominantly detected in the particulate fraction as a specific single band with the reasonable molecular mass of 110 kDa by anti-GFP polyclonal antibody. Treatment with TPA induced the translocation of MARCKS-GFP from the particulate to the cytosol fraction. No degraded products of MARCKS-GFP were found in the control cells or even in the cells treated with TPA. We examined the expression levels of endogenous and GFP-fused MARCKS. The cells expressing MARCKS-GFP were homogenated after the TPA treatment (1 μM, 10 min), and then the samples were analyzed by immunoblotting using anti-phospho-MARCKS (anti-pp-3) antibodies. Alternatively, the homogenate of cells expressing MARCKS-GFP was treated with TPA in the presence of [γ-32P]ATP, and then the expression levels were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The GFP-tagged MARCKS was expressed at a level between 7- and 10-fold that of endogenous MARCKS (data not shown).

**Translocation of MARCKS-GFP Induced by TPA and UTP**—The translocation of MARCKS after the activation of PKC was visualized by monitoring GFP fluorescence in living cells expressing MARCKS-GFP. Intense fluorescence of MARCKS-GFP was observed on the plasma membrane of the transfected CHO-K1 cells in vivo (Fig. 3). The activation of PKC by 100 nM TPA induced a rapid translocation of MARCKS from the plasma membrane to the cytosol (Fig. 3A) within 20 s after the stimulation. Fluorescence gradually accumulated in the perinuclear region, remained there for at least 60 min after treatment with TPA, and did not return to the plasma membrane. The activation of PKC through purinergic receptors by UTP (100 μM) induced a rapid and reversible translocation of MARCKS-GFP (Fig. 3B). Within 10 s after UTP stimulation, MARCKS-GFP was translocated from the plasma membrane to the cytosol, then from the cytosol to the plasma membrane, and was restored within 1 min to a state similar to that before stimulation. To investigate whether or not the translocation of MARCKS is induced by its phosphorylation by PKC, we examined the effects of staurosporine, a potent inhibitor of protein kinases, on the translocation of MARCKS-GFP. TPA-induced translocation was completely blocked by pretreatment with 1 μM staurosporine (Fig. 3C). Similarly, UTP-induced translocation was inhibited by staurosporine (data not shown).

**Immunocytochemical Analysis of MARCKS-GFP and MARCKS—GFP fluorescence showed that MARCKS-GFP was mainly found on the plasma membrane and faintly in the cytoplasm (Fig. 4a), as seen in the living cells. However, no immunoreactivity for phospho-MARCKS was detected in the untreated CHO-K1 cells expressing MARCKS or MARCKS-GFP when anti-phospho-MARCKS (anti-pp-3) antibodies that recognize the three putative PKC phosphorylation sites were used (Fig. 4, b and c). After TPA treatment, intense immunoreactivity for phospho-MARCKS was observed in the cytosol of the CHO-K1 cells expressing MARCKS or MARCKS-GFP (Fig. 4, e and f). The intracellular distribution of the GFP fluorescence of MARCKS-GFP corresponded with the immunoreactivities for phospho-MARCKS in TPA-treated CHO-K1 cells expressing MARCKS or MARCKS-GFP (Fig. 4, d-f).
tein—To examine the mechanism of the translocation of MARCKS-GFP induced by PKC activation, we constructed cDNAs for mutants of MARCKS-GFP. In BS580 (m3), three PKC phosphorylation sites (Ser-152, Ser-156, and Ser-163) were mutated to alanine, as described above (Fig. 1). BS580 was not expected to be phosphorylated by PKC. The m3 mutant (BS580) was only seen on the plasma membrane of the transfected CHO-K1 cells, and TPA failed to induce the translocation of this mutant (Fig. 5A). In BS644 (G2A), the N-terminal glycine was replaced with alanine, resulting in a non-myristoylated molecule. Non-myristoylated MARCKS-GFP was present throughout the cytoplasm, including the nucleoplasm, and no significant change in its localization was seen after TPA treatment (Fig. 5B). Stimulation with UTP that also activates PKC through G protein-coupled purinergic receptors also failed to induce the translocation of both m3 and G2A mutants of MARCKS-GFP (data not shown).

Translocation of MARCKS-GFP after H₂O₂ Treatment—We examined whether translocation of MARCKS-GFP is induced by treatment with hydrogen peroxide (H₂O₂) that activates δ-PKC by its tyrosine phosphorylation but does not translocate δ-PKC (46, 47). The basal kinase activity of transfected δ-PKC-GFP, which was immunoprecipitated with the anti-δ-PKC antibody, was 2–2.5-fold higher than that of endogenous δ-PKC.
The mutant MARCKS-GFP did not show translocation by H$_2$O$_2$ treated BS644. TPA at 100 nM did not induce the translocation of BS644 (Fig. 5A). The kinase activities of both endogenous and transfected δ-PKC-GFP were increased 3-fold after treatment with 5 mM H$_2$O$_2$ for 10 min. However, the same treatment with H$_2$O$_2$ did not alter the localization of MARCKS-GFP, as seen in Fig. 6A.

**The Phosphorylation of MARCKS-GFP and Its Mutants Treated with TPA and H$_2$O$_2$—**The phosphorylation of MARCKS-GFP and its mutants (m3 and G2A) was examined by immunoblotting after TPA and H$_2$O$_2$ treatment (Fig. 7) using anti-phospho-MARCKS (anti-pp-3) antibody. Wild-type MARCKS-GFP was predominantly localized in the particulate fraction, and phosphorylated MARCKS-GFP was not detected before stimulation with TPA. TPA treatment translocated MARCKS-GFP from particulate to cytosolic fraction, and the translocated MARCKS-GFP in the cytosol was phosphorylated (Fig. 7A). The m3 mutant MARCKS-GFP was also phosphorylated before stimulation, whereas the G2A mutant was slightly phosphorylated without stimulation. The expression level of the two mutants was similar to that of wild-type MARCKS-GFP (approximately 7–10-fold that of endogenous MARCKS-GFP). Neither the localization of G2A in the cytosol fraction nor that of m3 mutant in the particulate fraction was altered by TPA treatment. Furthermore, neither mutant was phosphorylated by TPA treatment. In contrast, H$_2$O$_2$ treatment caused neither PKC-dependent phosphorylation nor translocation of either wild-type or m3 mutant of MARCKS. It is noteworthy that G2A mutant MARCKS-GFP was phosphorylated after treatment with H$_2$O$_2$ (Fig. 7B), although G2A mutant MARCKS-GFP did not show translocation by H$_2$O$_2$ treatment.

We further examined the phosphorylation of wild-type MARCKS-GFP and G2A mutant MARCKS-GFP by δ-PKC in vitro using autoradiography and immunoblotting with anti-phospho-MARCKS (anti-pp-3) antibody. δ-PKC, which was immunoprecipitated from non-treated CHO-K1 cells overexpressed by PKC targeting for MARCKS phosphorylation. We investigated the phosphorylation-dependent interaction of MARCKS with macromolecule within the cells. Although both phosphorylated and unphosphorylated G2A mutants were distributed throughout the cytoplasm of the transfected cells, wild-type MARCKS accumulated around the nucleus after TPA treatment (Fig. 9A). In the photobleaching experiments, we measured the time necessary for the recovery of fluorescence in a square that was photobleached by a 488-nm argon laser. Photobleaching of wild-type MARCKS-GFP in the perinuclear region of the TPA-treated cell abolished the fluorescence of GFP in the square, and the fluorescence slowly recovered. The phosphorylated wild-type MARCKS-GFP had a recovery time of more than 5 min (Fig. 9A). In contrast, photobleaching of G2A-GFP in a squared area of H$_2$O$_2$-treated cells reduced the GFP fluorescence, not only of the squared area, but also of all the cytoplasm. The fluorescence in the square rapidly recovered to a level similar to that of the surrounding un-
bleached areas. The phosphorylated G2A mutant had a recovery time of less than 20 s (Fig. 9B). The GFP fluorescence in the nucleoplasm, however, was not altered 20 s after the bleaching of the cytoplasm and gradually decreased (Fig. 9B). Five min after the bleaching, the fluorescence in the nucleus was decreased to a level similar to that of perikarya.

Effects of Translocation and Phosphorylation of MARCKS on Actin Cross-linking Activity—To elucidate the functional significance of the translocation and phosphorylation of MARCKS, F-actin was stained with rhodamine-labeled phalloidin in CHO-K1 cells expressing MARCKS-GFP and its G2A mutant after the treatment with TPA and H2O2. In CHO-K1
cells expressing MARCKS-GFP, F-actin was densely found on the plasma membrane, and few stress fibers were seen in the cytoplasm, whereas stress fibers were evident in the surrounding untransfected CHO-K1 cells (Fig. 10b). TPA treatment significantly decreased the amount of F-actin on the plasma membrane, and stress fibers were clearly seen in the cytoplasm (Fig. 10f). H₂O₂ treatment did not change the localization of F-actin in CHO-K1 cells expressing MARCKS-GFP (Fig. 10j). Considerable numbers of stress fibers were seen in CHO-K1 cells expressing G2A-GFP (Fig. 10c). There were more abundant stress fibers in the G2A-transfected cells than in the surrounding untransfected cells. TPA treatment did not alter the localization of F-actin in CHO-K1 cells expressing G2A-GFP (Fig. 10c); however, H₂O₂ treatment slightly decreased the number of stress fibers in the cytoplasm (Fig. 10f).

DISCUSSION

Recent studies on PKC translocation using GFP-fusion protein revealed that each PKC subtype has a unique targeting mechanism that depends on various stimulations. The first visualization of PKC translocation, by Sakai et al. (13), showed that γ-PKC translocates to plasma membrane within 5 min in response to TPA stimulation, whereas Ca²⁺ ionophore induced a rapid and reversible translocation. Rapid and reversible translocation of PKC was also observed after the stimulation of cell surface receptors, such as metabotropic glutamate receptors and purinergic receptors that induce the breakdown of phosphatidylinositol (13, 14). Furthermore, stimulation with various fatty acids induced subtype-specific and stimulus-specific translocation of PKC (14). Ohmori et al. (47) also demonstrated that different targeting of δ-PKC through various activation processes of the enzyme caused distinct cellular responses. Recently, Wang et al. (48) reported different translocation of δ-PKC by phorbol esters and related compounds. Two tumor promoters, phorbol 12-myristate 13-acetate and 12-deoxyphorbol 13-monoesters, induced plasma membrane translocation of δ-PKC followed by slower nuclear translocation. In contrast, only nuclear translocation of δ-PKC was induced by similar PKC activators, bryostatin and 12-deoxyphorbol phenylacetate, that have antipromoting effects (48). These results also suggested that distinct pharmacological effects of the drugs may be exerted due to the different translocation of the PKC subtype.

Since the first report of PKC translocation in living cells appeared (13), it has been thought that translocation from cytosol to the particulate fraction is necessary for PKC activation. PKC, however, translocates to various compartments of cells and can be activated without translocation by hydrogen peroxide. These findings suggested the idea that PKC translocates to a specific compartment in order to interact with its specific substrate as well as binding to lipid activators on the membrane. It is also plausible that PKC activation without translocation may cause phosphorylation of PKC substrate in the cytoplasm. Thus, analysis of the temporal and spatial targeting of PKC is now the most attractive way of studying the multiple functions of PKC.

MARCKS is a prominent substrate for PKC and is also known to be a multifunctional protein controlling actin cross-linking activity or calmodulin concentration (15–23). MARCKS is localized on the membrane and shuttles between membrane and cytoplasm in a PKC phosphorylation-dependent manner (40, 49). The binding of MARCKS to the membrane is regulated by two determinants: insertion of its myristoylated N terminus into the lipid bilayer and electrostatic interaction of the cluster of basic residues in the effector domain with the acidic lipids of the membrane (35–38). Phosphorylation of MARCKS by PKC induces translocation of MARCKS from membrane to cytosol by introducing negative charges into the basic cluster (27, 36, 37, 40, 42).

Immunoblot analysis Fig. 2) showed that MARCKS-GFP was not degraded before or after the TPA treatment. Because anti-pp antibody reacted with wild-type MARCKS only after TPA treatment and did not react with the m₃ mutant of MARCKS, anti-pp antibody specifically recognizes phosphorylated MARCKS. Furthermore, immunocytochemical studies revealed that the GFP fluorescence fused to MARCKS was colocalized with phospho-MARCKS immunoreactivity after TPA treatment (Fig. 4). These results indicate that the phosphorylation of MARCKS can be detected by anti-phospho-MARCKS (anti-pp) antibody, and GFP fluorescence can be used as a marker for native MARCKS translocation. In the present study, we first visualized the translocation of MARCKS from plasma membrane to cytoplasm in living cells. After TPA or UTP stimulation, which causes the translocation of cPKC and nPKC when expressed in CHO-K1 cells from cytosol to membrane, MARCKS was phosphorylated, and the phosphorylated MARCKS was translocated from plasma membrane to cytoplasm. From the findings that the translocation was inhibited by staurosporine and that the mutation of PKC phosphorylation sites (m₃ mutant) in MARCKS completely blocked both translocation and phosphorylation of MARCKS, it is indicated that MARCKS translocation is induced by the PKC-dependent phosphorylation of serine residues in the basic cluster. TPA induced a rapid translocation of MARCKS to the cytoplasm and then a slower accumulation in the perinuclear region, which is...
Importance of PKC Targeting for MARCKS Phosphorylation

Although most PKC subtypes except for \( \xi \)-PKC were translocated to plasma membrane in response to phorbol ester (3), the intracellular localization and translocation varied among PKC subtypes in various cell types. TPA-induced translocation of \( \alpha \)-PKC to nuclear membrane in addition to plasma membrane was reported in fibroblasts (52, 53), and the translocation of \( \beta \)-PKC cytoskeletal proteins, such as ankynin and microfilament, was also shown in \( T \) lymphocyte (54) and cardiac myocytes (55). As shown in Fig. 10, the increase in F-actin in cells expressing G2A mutant MARCKS-GFP strongly suggests that the phosphorylation of the mutant MARCKS-GFP was observed, suggesting that PKC subtypes in CHO-K1 cells were not translocated to F-actin by TPA treatment. The association of \( \epsilon \)-PKC with F-actin has been reported (56, 57), but TPA did not appear to translocate any PKC subtype to F-actin in the present study. Perhaps, it is because only \( \alpha \), \( \beta \)II, \( \delta \), and \( \xi \)-PKCs but not \( \epsilon \)-PKC were expressed endogenously in CHO-K1 cells (58).

Mutation of the N-terminal glycine results in a non-myristoylated form of MARCKS that does not bind to the membrane, as described previously (39). The present results also demonstrated that non-myristoylated MARCKS fused to GFP was localized in the cytoplasm but not on the membrane. The G2A mutant was neither phosphorylated nor translocated when TPA was applied to the cells expressing the mutant MARCKS, as described previously (39). It is possible that non-myristoylated MARCKS is no longer a good substrate for PKC after its mutation. However, G2A mutant was phosphorylated in vitro by the immunoprecipitated \( \delta \)-PKC as much as the wild-type MARCKS (Fig. 8). Neither of two activation processes of PKC by TPA and \( H_2O_2 \) caused significant differences in the level of the phosphorylation between wild-type and mutant MARCKS. Because TPA activates PKC by translocating the enzyme from cytoplasm to membrane, and \( H_2O_2 \) activates PKC without translocation (47), we examined the phosphorylation of wild-type and mutant MARCKS in vitro after two extracellular stimulations that activate PKC by different targeting. As shown in Fig. 7, wild-type MARCKS was phosphorylated only when the cells were treated with TPA, but non-myristoylated MARCKS was phosphorylated only when treated with \( H_2O_2 \). The level of phosphorylation was analyzed by comparing densitometric intensity between phosphorylated MARCKS-GFP by anti-pp3 antibody and total MARCKS-GFP by anti-GFP antibody. The level of phosphorylation of G2A mutant MARCKS-GFP in the cytosol fraction by \( H_2O_2 \) (arbitrary units, 0.91) appeared to be slightly lower than that of wild-type MARCKS-GFP in the cytosol fraction by TPA (arbitrary units, 1.12). However, because the level of phosphorylation of wild-type MARCKS-GFP in the particulate fraction was significantly low (arbitrary units, 0.33), there was no significant difference in the phosphorylation level of the total fractions between wild-type and mutant (G2A) MARCKS-GFP (arbitrary units, 0.788 and 0.794, respectively). The present results suggested that PKC that has been translocated to membrane can phosphorylate membrane-bound MARCKS (wild-type) but not cytosolic MARCKS (G2A), whereas PKC that is activated in the cytoplasm without translocation can phosphorylate cytosolic MARCKS (G2A) but not membrane-bound MARCKS (wild-type).

In conclusion, the targeting of PKC to a specific cellular compartment is an important event to phosphorylate a substrate that is localized in that specific compartment; the different targeting of PKC thus results in different cellular responses.

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Importance of Protein Kinase C Targeting for the Phosphorylation of Its Substrate, Myristoylated Alanine-rich C-kinase Substrate
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