Protein Kinase C δ Activates the MEK-ERK Pathway in a Manner Independent of Ras and Dependent on Raf*

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Although the involvement of protein kinase C (PKC) in the activation of the mitogen-activated protein (MAP) kinase pathway has been implicated through experiments using 12-O-tetradecanoylphorbol-13-acetate (TPA), there has been no direct demonstration that PKC activates the MAP kinase pathway. A Raf-dependent intact cell assay system for monitoring the activation of MAPK/ERK kinase (MEK) and extracellular signal-related kinase (ERK) permitted us to evaluate the role of PKC isotypes in MAP kinase activation. Treatment of cells with TPA or epidermal growth factor resulted in the activation of MEK and ERK. The activation of the MAP kinase pathway triggered by epidermal growth factor was completely inhibited by dominant-negative Ras (RasN17), whereas the activation triggered by TPA was not, consistent with previous observations. The introduction of an activated point mutant of PKCδ, but not PKCα or PKCε, resulted in the activation of the MAP kinase pathway. The activation of MEK and ERK by an activated form of PKCδ requires the presence of c-Raf and is independent of RasN17. These results demonstrate that activation of PKCδ is sufficient for the activation of MEK and ERK and that the pathway operates in a manner dependent on c-Raf and independent of Ras.

Mitogen-activated protein kinases (MAP1 kinases; ERK1 and ERK2) are common intermediates in intracellular signaling cascades involved in diverse cellular functions including growth and differentiation (1, 2). The activation of MAP kinases requires the dual-phosphorylation of Thr and Tyr residues by activating kinases, MAP kinase kinases (MEK1 and MEK2). The activity of MAP kinase kinases is also regulated by phosphorylation, and the responsible kinases have been identified (3, 4). c-Raf is a MAP kinase kinase that directly phosphorylates and activates MEK1 and MEK2 (5, 6). The extracellular stimuli that activate MAP kinases include insulin, EGF, platelet-derived growth factor, nerve growth factor, serum, phorbol esters, nicotine, okadaic acid, and activators of oocyte maturation. The signaling pathway involved in the activation of ERKs has been intensively studied for c-Raf and Ras, and the mechanism of their activation has been analyzed in detail. This has led to an understanding of the presence of a linear array of signaling pathway initiated by tyrosine kinases to activate Ras, c-Raf, MEKs, and ERks (7). However, there remain many fundamental questions concerning the mode of activation of c-Raf and the signaling pathway from extracellular stimuli to the Raf-MEK-ERK pathway.

One of these questions concerns the role of protein kinase C (PKC), a family of enzymes activated through a pathway involving a diverse set of lipid metabolites activated by phospholipase C, phosphatidylinositol 3-kinases, and other molecules (8, 9). Treatment of cells with TPA or EPDA resulted in the activation of c-Raf (10) and MAP kinase (11–13) within minutes, suggesting the involvement of PKC in the signaling pathway leading to MAP kinase activation. Although these observations suggest a link between PKC and MAP kinase activation, there has been no direct demonstration that some members of the PKC family actually activate the pathway. Furthermore, the target of PKC in the activation of the MAP kinase pathway, if PKC is involved, remains to be clarified. The involvement of Ras (14–22) and c-Raf (19, 21, 23–28) in the TPA-induced activation of MAP kinases has been reported with rather paradoxical results.

Recently, G protein βγ has been reported to be involved in the Ras-dependent activation of MAP kinases (29–31), and Gα protein α has been reported to be involved in Ras-independent activation of MAP kinases (32). Furthermore, a protein tyrosine kinase, PYK2, has also been reported to be involved in Ras-independent MAP kinase activation (33). The involvement of PKC in this novel pathway has also been suggested.

However, the above studies used TPA, raising the fundamental question of whether PKC is actually involved. The presence of a set of cellular proteins that bind to phorbol esters and the observation that their activities are modulated by phorbol esters also raises the question of whether the effect of TPA on MAP kinases actually involves PKC. Cellular phorbol ester receptors other than PKC include Ras-activating guanine nucleotide exchange factor and Rac-GTPase activating protein (34, 35). In the present study, we addressed the question of whether PKC is actually involved in MAP kinase activation by TPA, and, if it is, which PKC isotype is involved. Using a series of PKC kinase-knockout mutants and mutants with constitutive kinase activity, we show that PKCδ is actually involved in the signaling pathway from TPA to Raf-MEK-ERK activation that operates in a Ras-independent manner.

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1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; MBP, myelin basic protein; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; TRE, TPA response element; CAT, chloramphenicol acetyltransferase.
EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was obtained from Amersham Corp. TPA was from Sigma, phosphatidylinerine was from Funakoshi, and 2-mercaptoethanol was from Nacalai Tesque. Oligopeptide MBP-IV, 14, corresponding to residues 41–60 of MBP, was synthesized and purified as described previously (36).

Plasmids—ERK1 (37, 38) cDNA was obtained from rat brain mRNA by reverse transcription-polymerase chain reaction. Polymerase chain reaction was performed by using the 5′ primer 5′-CTGATTACCAT-CACGCTGAGATGCGC-3′ and the 3′ primer 5′-GAGAATCTTCTT-TAGTGGAGCCTG-3′, corresponding to the 5′ and 3′ coding regions of rat ERK1. EcoRI restriction sites were generated at both ends of the cDNA. MEK1 (39) cDNA was obtained from mouse brain mRNA by reverse transcription-polymerase chain reaction. Polymerase chain reaction was performed using the 5′ primer 5′-ATGGATCCCTGCAA-GATGCCCAGAAGA-3′ and the 3′ primer 5′-GGTGGATCCCTTAAAG-GCTCAGATCTGG-3′, corresponding to the 5′ and 3′ coding regions of mouse MEK1. BamHI restriction sites were generated at both ends of the cDNA. c-Raf cDNA (40) was provided by the Japanese Cancer Research Resources Bank.

Transfection—The cDNA constructs were ligated into the EcoRI site of the bacterial expression vector pGEX-3X. The construct was transformed into Escherichia coli strain XL1-Blue, and recombinant GST-MEK1 protein was expressed and purified as reported (50).

Protein Kinase C—Expression and purification of GST-ERK1—Rat ERK1 cDNA was cloned into the EcoRI site of the bacterial expression vector pGEX-3X to yield pGEX-3X-ERK1. The construct was transformed into E. coli strain strain XL1-Blue, and recombinant GST-ERK1 protein was expressed and purified as reported (50).

Western Blot Analysis—Following SDS-polyacrylamide gel electrophoresis, the separated proteins were electroblotted electrophotochemically transferred to a polyvinylidene difluoride membrane, and the membrane was soaked in phosphate-buffered saline containing 5% skimmed milk for 1 h at room temperature. ERK and MEK were detected using polyclonal antibodies raised against rat ERK (06 (325) (Upstate Biotechnology, Inc.), respectively. Peroxidase-labeled horseradish peroxidase-conjugated sheep anti-rabbit or mouse Ig (Amersham) was used as a secondary antibody for signal detection by the ECL detection system (Amersham).

RESULTS

A Raf-dependent Pathway for MEK and ERK Activation in Intact Cells—To address the question of whether PKC is actually involved in the activation of MAP kinase and, if it is, how PKC activates MAP kinase, we devised an assay system in COS cells where the effect of exogenous proteins in the activation of MEK1 and ERK1 could be evaluated. Introducing tagged MEK1 into COS cells, stimulating the cells with TPA or EGF, immunoprecipitating the tagged MEK1, and measuring the MEK1 activity in vitro using recombinant GST-ERK1 as a substrate permitted us to monitor the activation of MEK1 in...
response to TPA. Fig. 1A shows that treatment of cells with TPA or EGF results in 5–6-fold activation of MEK1 within 10 min. Furthermore, the co-expression of c-Raf potentiates the activation of MEK1 by TPA. The potentiation of MEK1 by c-Raf co-expression was also seen following EGF treatment, although the response was weaker. These results are consistent with previous observations that TPA-stimulated ERK activation is potentiated by the overexpression of c-Raf (19) and that the N-terminal fragment of c-Raf or the point mutant of c-Raf (K375W) suppresses the TPA-induced mobility shift of ERKs (21, 23, 28). Furthermore, the results demonstrate that this system can be used for the analysis of the Raf-dependent pathway for MEK1 and ERK1 activation.

To examine the involvement of Ras in the above system, we used a dominant-negative Ras mutant, Ki-RasN17. As shown in Fig. 1A, RasN17 only slightly suppresses the TPA-induced activation of MEK1, whereas it completely suppresses the EGF-induced activation of MEK1. Similar results were also obtained for ERK1 in COS cells and NIH3T3 cells, as shown in Fig. 1. B and C, where tagged ERK1 was used instead of tagged MEK1, and the upward shift in the electrophoretic mobility of tagged ERK1 was evaluated using an anti-ERK antibody. These results confirm the previous observations that the activation of ERKs by TPA depends on c-Raf but not on Ras (19, 21).

**Dominant-negative Action of PKC Kinase-Knockout Mutants on TPA-induced MEK Activation**—Since PKC is the major receptor for phorbol esters, PKC is the most obvious candidate for the substance that mediates the TPA-induced activation of MEK and ERK shown above. However, there have been few in vivo demonstrations that any PKC members are actually involved in the signaling pathway. Only PKCβ1 has been reported to potentiate the TPA-induced activation of c-Raf when overexpressed in insect cells (24). To address this issue, we next examined the effect of a series of kinase-knockout point mutants of PKC isozymes expressed ubiquitously in a wide variety of cells, including COS and NIH3T3 cells.

We introduced a series of PKC kinase-knockout mutants into COS cells and evaluated their effects on TPA-induced MEK1 activation. The kinase-knockout mutants were designed to have "activated conformation" by introducing mutation(s) into the pseudosubstrate region (see "Discussion"). Fig. 2 shows that the overexpression of the kinase-knockout mutants of PKCa, PKCβ, and PKCε all produce inhibition of the TPA-induced activation of MEK1 in a dose-dependent manner. However, this inhibition is incomplete at the highest DNA amount.
when the amount of the mutants was more than 100 times relative to their corresponding counterparts (Fig. 2D). The results suggest that PKC and/or its homologue is involved in the signaling pathway from TPA to MEK1 activation. However, the results raise the question of the specificity of the kinase-knockout mutants on the action of each respective PKC isotype.

**Constitutively Active PKCδ Activates the MEK-ERK Pathway**—To examine the specificity of the action of each PKC isotype directly, we next tried to determine the effect of the overexpression of PKC members on the TPA-induced activation of MEK1 and ERK1. However, we failed to detect any significant effect (data not shown), suggesting that the amount of endogenous PKC in COS and NIH3T3 cells is sufficient to mediate the TPA-induced activation of MEK1 and ERK1. Thus, we next constructed a series of constitutively active PKC mutants (Fig. 3A).

PKCa (α22A/A25E) has two point mutations in its pseudosubstrate region. The kinase activity of the mutant was evaluated after immunoprecipitation and showed clearly that PKCa (α22A/A25E) is fully active in the absence of cofactors (Fig. 3B). The introduction of the PKCo mutant with reporter plasmids containing TPA response elements (TRE-luciferase) resulted in gene expression without TPA stimulation (data not shown), consistent with the results of the in vitro kinase assay. PKCδ (DR144/145A) also contains two point mutations and is fully active without cofactors (Fig. 3C). This is consistent with the previous observation that the introduction of PKCδ (DR144/145A) into NIH3T3 cells results in the activation of reporter gene expression (TRE-tk-CAT) without any stimuli (44). PKCe (εA159E) contains a point mutation in its pseudosubstrate region. The in vitro kinase assay using immunoprecipitated PKCe failed because of the absence of an antibody able to immunoprecipitate PKCe and monitor its kinase activity in a cofactor-dependent manner. However, the same mutant has been shown to be apparently constitutively active when monitored in terms of reporter gene expression (45). The overexpression of the respective wild-type or constitutively active mutant of PKCa, δ, or ε was confirmed by Western blot analysis (Fig. 3A).

We next overexpressed the constitutively active PKC mutants and examined their effects on MEK1-ERK1 activation. Overexpression of PKCa, PKCe (α22A/A25E), and PKCe (εA159E) failed to activate MEK1 (Fig. 4A) or ERK1 (Fig. 4, B and C). Another constitutively active mutant of PKCe, the kinase domain, also failed to activate MEK1 under our assay conditions (data not shown), although the same mutant induces TRE-CAT expression without any stimuli (51). However, the overexpression of PKCδ resulted in a 3-fold enhancement of MEK1 activation, and the overexpression of its constitutively active mutant, PKCδ (DR144/145A), resulted in an 8-fold enhancement of MEK1 activation (Fig. 4A). Although the amount of each construct of PKCa, δ, or ε cannot be directly compared (Fig. 3A), experiments using less (1 µg) and more (5 µg) amounts of the expression vector gave similar results (data not shown). The overexpression of PKCδ (DR144/145A) also resulted in the activation of ERK1 (Fig. 4: B, COS cells, and C, NIH3T3 cells). The overexpression of wild-type PKCδ, but not other PKC isotypes, resulted in a slight activation (30% mobility shift) of ERK1 in NIH3T3 cells (Fig. 5, B and C). These results clearly show that PKCδ is a likely mediator of the action of TPA in MEK1 and ERK1 activation in the assay system used in the present study.

**PKCδ-mediated MEK/ERK Activation Requires c-Raf But Is Independent of Ras**—The ability of the PKCδ mutant, PKCδ (DR144/145A), to activate MEK1 and ERK1 prompted us to examine the involvement of Ras in the signaling pathway. As shown in Fig. 5A, PKCδ (DR144/145A) cannot activate MEK1 in the absence of c-Raf, whereas c-Raf co-expression results in MEK1 activation. Furthermore, MEK1 activation by PKCδ (DR144/145A) is not inhibited by RasN17, PKCδ (DR144/145A) activates ERK1, as shown in Fig. 5, B and C. The activation of ERK1 requires the presence of c-Raf and is independent of the presence of RasN17 (Fig. 5, B and C). Thus, PKCδ (DR144/145A) activates MEK1 and ERK1 in a manner dependent on c-Raf. Furthermore, activation occurs in the presence of RasN17.

**DISCUSSION**

The effect of TPA on the tyrosine phosphorylation of the protein p42 was observed before the discovery of MAP kinases (52–54), and the effect of TPA on the activation of MAP kinase was also reported in the original identification of MAP kinases.
Since TPA directly activates PKC, it has been implicated in the signaling pathway from TPA to MAP kinase activation. However, it is now evident that there are proteins in addition to PKC isotypes that are potential cellular phorbol ester receptors. These include Vav, a guanine-nucleotide exchange protein known to be involved in the activation of Ras (34), and n-chimaerin, a GTPase activating protein, which inactivates another small G-protein, Rac (35). In the present study, we used an intact cell assay system in which the TPA-induced activation of MEK1 and ERK1 occurs in a Raf-dependent manner and demonstrated that the kinase-knockout mutants of PKCα, PKCδ, and PKCe inhibit the TPA-induced activation of MEK1, indicating that PKC or its homologues are involved in the signaling pathway from TPA to MEK-ERK. More importantly, we showed that a constitutively active mutant of PKCδ causes the activation of MEK1 and ERK1. This is one of the most important points of the present study, since it excludes the possibility that TPA-induced MAP kinase activation requires phorbol ester receptors other than PKC isotypes. Quite interestingly, the constitutively active mutant of PKCδ failed to activate MEK1 and ERK1 in a similar assay system. These results show that PKCδ is actually involved in and is sufficient for the activation of MEK and ERK. Furthermore, PKCδ activates MEK1 and ERK1 in the presence of RasN17, indicating that the signaling pathway operates in a Ras-independent manner.

The constitutively active form of PKC was reported in the original identification of PKC (55). Deletion of the regulatory domain of PKCα or PKCβ allows them to be constitutively active; their expression results in the activation of c-fos promoter and TRE elements and the induction of *Xenopus oocyte* maturation (51, 56, 57). However, the regulatory domain of PKC has also been suggested to be involved in substrate recognition, and its deletion might result in the generation of a
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105 cells) cultured on 15-mm dishes were co-transfected with tag-ERK1 and/or H9262 (2/H9262) together with the indicated amount (0, 0.1, 0.3, 1, or 3 g/H9262) of tag-MEK1 (5 g/H9262) or H9251 with the respective constitutively active mutant of PKC. PKCa (aR22A/A25E), which shows completely cofactor-independent activity (Fig. 3B) and induces reporter expression (data not shown). The constitutively active mutant of PKCδ (DR144/145A) has been shown previously to be active, as judged by its induction of TRE-CAT expression (44). In the present study, we showed that this mutant does, in fact, have cofactor-independent activity (Fig. 3C). Although we failed to confirm that PKCe (eA159E) shows cofactor-independent activity in the in vitro kinase assay, the mutant was shown to be active, as judged by its activation of reporter genes such as TRE-CAT, NFAT-CAT, and NFκB-CAT (45), strongly supporting the idea that this mutant is active in vivo. This excludes the possibility that the failure of these mutants to activate MEK and ERK is due to the fact that they are not fully active. One possible explanation for the failure of PKCa and PKCe to activate MEK and ERK is that they overactivate MEK and ERK and cause their own down-regulation. However, this possibility is unlikely since smaller amounts of DNA also failed to activate ERK1 (Fig. 4, B and C). Thus, the failure of the PKCa and PKCe mutants to activate MEK and ERK may reflect the intrinsic properties of the PKC isotypes.

The dominant-negative effects of the kinase-knockout mutants of PKCa, PKCδ, and PKCe on the TPA-induced activation of MEK1 might suggest the involvement of these PKC members and/or other PKC homologues. However, the dominant-negative effect was incomplete at the highest level of the kinase-knockout mutant expression (Fig. 2D). Furthermore, there was no isozyme specificity making a clear contrast to the results obtained by constitutively active mutants. This might suggest that the dominant-negative effect was caused by the competition of TPA rather than their target protein(s). In addition, TPA might activate MEK and ERK through a pathway that does not involve PKC.

There are several reports that focus on PKC isotypes and their potential for MAP kinase activation. Marquardt et al. (24) have reported that the co-expression of PKCβ1 and c-Raf in insect cells results in a potentiation of the TPA-induced activation of c-Raf when the activity of the immunoprecipitated c-Raf was evaluated by an in vitro GST-MEK activation assay. It has been reported that purified PKCa phosphorylates c-Raf and activates autokinase activity (62, 63). Note that the authors did not examine the activity of c-Raf in terms of MEK activation, and that there is a report that purified brain PKC (cPKC mixture) phosphorylates c-Raf in vitro and activates its autokinase activity but does not activate its MEK phosphorylation activity (64). The results of Marquardt et al. (24) showing that PKCδ1 potentiates the TPA-induced activation of c-Raf to phosphorylate MEK suggests the involvement of PKCδ1 in TPA-induced c-Raf activation. However, the use of TPA does not directly show that PKCδ1 is sufficient to activate c-Raf. The present demonstration using constitutively active mutants of PKC isotypes that PKCδ but not PKCa or PKCe activates MEK1 and ERK1 clearly indicates that the activation of PKCδ is sufficient to activate the MEK-ERK pathway and strongly
c-Raf has been shown to phosphorylate and activate MEK directly (5). The overexpression of c-Raf potentiates the TPA-induced activation of MEK1 and ERK1 (Fig. 1A) (19), suggesting the involvement of c-Raf in the signaling pathway from TPA to MEK-ERK. Consistent with this is the report that the N-terminal fragment of c-Raf, or a point mutation of c-Raf (K375W), inhibits the TPA-induced activation of MAP kinase in COS cells and in 293 cells (21, 23, 27, 28). Furthermore, the activation of c-Raf in TPA-treated COS cells has been shown in vitro using immunoprecipitated c-Raf and MEK as substrates (19). However, there is also a report that a kinase-knockout mutant of c-Raf fails to inhibit the phorbol 12,13-dibutyrate-induced activation of MAP kinase in LA-90 cells (25). Several lines of evidence indicate that PKC phosphorylates c-Raf in TPA-treated cells. In vitro, PKCα phosphorylates c-Raf, presumably at site S499 (63), and activates c-Raf in terms of a kinase activity against a peptide spanning the c-Raf autophosphorylation site. The phosphorylation of c-Raf at S499 occurs in vivo when A293 cells are treated with TPA. TPA treatment of insect cells co-expressing PKCα and c-Raf results in the activation of a c-Raf kinase activity against a peptide spanning the c-Raf autophosphorylation site, and the c-Raf S499A mutant is not activated under the same condition (63). These in vivo results confirm the notion obtained from in vitro results that PKCα phosphorylates c-Raf at S499 and activates its autokina- nase activity. Carroll and May reported that rat brain PKC (cPKC mixture) phosphorylates c-Raf at S497 and S619 and activates c-Raf kinase activity against histones III (65). How- ever, as mentioned before, there has been no direct demonstration that c-Raf phosphorylated by PKC has an increased capacity to activate MEK. It is not clear whether PKCδ directly activates c-Raf. Whether PKCδ interacts directly with c-Raf and activates its activity toward MEK remains the subject for future experiments.

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suggests that PKCδ is a likely activator of the MEK-ERK pathway.

There are apparently paradoxical observations about the role of Ras in the phorbol ester-induced activation of MAP kinases. For example, in PC12 cells and NIH3T3 cells, the TPA-induced activation of MAP kinases involves Ras (14, 16, 17, 33). On the other hand, RasN17 fails to inhibit TPA-induced MAP kinase activation in Rat 1 cells, COS cells, and 293 cells (18, 19, 21). It should be noted that these are all observations using TPA or phorbol 12,13-dibutyrate and not a direct examination of the role of PKC. Considering that PKC directly modulates effectors of Ras, the action of TPA might involve these proteins in addition to PKC. There is a report in which cell-free extracts of Xenopus oocytes were used to show that an active fragment of brain PKC (cPKC mixture) activates MAP kinases and is inhibited by RasN17 (26). This suggests the presence of a Ras-dependent pathway involving PKC isotypes such as PKCs (Fig. 5D).

Fig. 5. Constitutively active PKCδ activates MEK1 and ERK1 in a Ras-independent manner. A. activation of MEK1. COS1 cells (5.0 × 10⁶ cells) were co-transfected with tag-MEK1 (5 µg) cDNA expression plasmid together with the cDNA expression plasmids of c-Raf (5 µg), DR144/145A (1 µg), or RasN17 (5 µg) as indicated. After 24 h of culture on 10-cm dishes, the cells were harvested. tag-MEK1 was immunoprecipitated, and its activity was evaluated. Values represent the means of three independent experiments; bars, S.D. B and C. activation of ERK1. COS1 (B) or NIH3T3 (C) cells (1.8 × 10⁶ cells) cultured on 15-mm dishes were co-transfected with tag-ERK1 (2 µg) and MEK1 (1 µg) cDNA expression plasmids together with the cDNA expression plasmids of c-Raf (1 µg), PKCδ (1 µg), DR144/145A (1 µg), or RasN17 (2 µg) as indicated. tag-ERK1 was visualized using anti-ERK1 antibodies. The figure shows the results of typical experiments, and similar results were obtained in three separate experiments. D. schematic drawing of the proposed mechanism for MAP kinase activation by PKC.
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