Activation of Protein Kinase C Triggers Its Ubiquitination and Degradation†

ZHIMIN LU,1 DAVID LIU,2 ARMAND HORNIA,1 WAYNE DEVONISH,1 MICHELE PAGANO,2 AND DAVID A. FOSTER1**

Department of Biological Sciences, Hunter College and the Graduate School of the City University of New York, New York, New York 10021,1 and Department of Pathology, New York University Medical Center, New York, New York 100162

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Tumor promotion by phorbol esters involves the selective amplification of cells previously mutated in an appropriate growth-stimulatory gene (3, 17). Phorbol esters exert their effects on the protein kinase C (PKC) family of genes, which consists of genes that encode at least nine distinct isoforms that are responsive to tumor-promoting phorbol esters (9). Phorbol esters first activate phorbol ester-responsive PKC isoforms, but upon prolonged treatment, these isoforms are proteolytically degraded (16). Using a cell culture model system in which cells overexpressing c-Src were transformed by phorbol ester treatment, we recently demonstrated that the tumor-promoting effect of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) on these cells was due to the depletion of PKC δ (7). These data suggested that PKC δ may function as a tumor suppressor. Consistent with this hypothesis, PKC δ was inactivated by tyrosine phosphorylation in cells transformed by v-Src and v-Ras (2). Thus, regulation of PKC δ at the level of activity and expression may be a very important cell growth control mechanism.

PKC α has been reported to become ubiquitinated in response to brystatin I, an activator of PKC that prevents tumor promotion in mouse skin by TPA (6). The ubiquitin-proteasome pathway is a nonlysosomal degradation system that controls the timely destruction of cell cycle-regulatory proteins, including the tumor suppressor p53; the cyclin-dependent kinase inhibitor p27; the cyclins; the oncogene products c-Myc, c-Jun, and c-Fos; and the transcription factors NF-κB and E2F (reviewed in reference 13). This pathway involves the covalent tagging of proteins with ubiquitin, followed by proteasome-mediated degradation of tagged proteins. Conjugation of ubiquitin to substrate proteins requires three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Both the E2 and E3 proteins belong to large families of proteins, and it is believed that different combinations of E2 proteins with different E3 ligases define a high substrate specificity. In this study, we have investigated the role of the ubiquitin-proteasome pathway in the downregulation of PKC isoforms in response to the tumor-promoting phorbol ester TPA.

MATERIALS AND METHODS

Cells and cell culture conditions. Rat 3Y1 cells or rat 3Y1 cells expressing either v-Src or c-Src were maintained in Dulbecco’s modified Eagle medium supplemented with 10% bovine call serum (HyClone). Cell cultures were made quiescent by growing them to confluence and then replacing the medium with fresh medium containing 0.5% newborn calf serum for 1 day. Cells expressing the kinase-dead PKC α were generated as described previously (7). The kinase-dead PKC α clone was generated by a mutation to the ATP-binding site as described previously (15).

Materials. The PKC inhibitors staurosporine, bisindolylmaleimide II, rottlerin, and Go6976 were obtained from Calbiochem. Monoclonal antibodies for PKC α, ε, and ξ were obtained from Transduction Laboratories; a polyclonal antibody for PKC δ was obtained from Santa Cruz. A monoclonal antibody for ubiquitin was obtained from Zymed.

Cell lysate preparation and subcellular fractionation. Cells grew to approximately 90% confluence in 100-mm-diameter culture dishes and were then shifted to Dulbecco’s modified Eagle medium containing 0.5% serum for 24 h. Cells were washed three times with ice-cold isotonic buffer (phosphate-buffered saline, containing 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, and 4.2 mM Na₂HPO₄, pH 7.2). For subcellular fractionation, cells from 100-mm-diameter dishes were washed and then scraped into 2 ml of homogenization buffer (20 mM Tris-HCl [pH 7.5], 5 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 2 mM dithiothreitol, 200 μM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). Cells were then disrupted with 20 strokes in a Dounce homogenizer (type B pestle), and the lysate was centrifuged at 100,000 × g for 1 h. The supernatant

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† This paper is dedicated to Erwin Fleissner on the occasion of his retirement as the dean of sciences and mathematics at Hunter College.

* Corresponding author. Mailing address: Department of Biological Sciences, Hunter College and the Graduate School of the City University of New York, 695 Park Ave., New York, NY 10021. Phone: (212) 772-4075. Fax: (212) 772-5227. E-mail: foster@genectr.hunter .cuny.edu.
was collected as the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton X-100. After incubation for 30 min at 4°C, the suspension was centrifuged at 100,000 \( g \) for 1 h. The supernatant was collected as the membrane fraction. For whole-cell lysates, cells were treated with 3 ml of homogenization buffer containing 1% Triton X-100 followed by centrifugation at 100,000 \( g \) for 1 h. The supernatant was collected and used as the whole-cell lysate.

**Immunoprecipitation and Western blot analysis.**

Extraction of proteins from cultured cells was performed as previously described (7) with a modified buffer consisting of 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (12 mg/ml), aprotinin (20 \( \mu \)g/ml), 100 \( \mu \)M sodium vanadate, 100 \( \mu \)M sodium pyrophosphate, 1 mM sodium fluoride, 10 mM ethylmethylmaleimide, and 50 mM hemin. Cell extracts were clarified by centrifugation at 12,000 rpm, and the supernatants (1,500 \( g \) of protein/ml) were subjected to immunoprecipitation with anti-PKC \( \delta \), \( \alpha \), and \( \varepsilon \) antibodies. After overnight incubation at 4°C, protein A-agarose beads were added and left for an additional 3 h. Immunocomplexes were then subjected to Western blot analysis as described previously (7). Western blot analysis with antiubiquitin antibody was performed with modifications described by Avantaggiati et al. (1).

**RESULTS**

Proteasome inhibitors block depletion of PKC isoforms. To investigate whether the ubiquitin-proteasome pathway is involved in the downregulation of PKC in response to phorbol esters, we first examined the effect of proteasome inhibitors on TPA-induced PKC depletion. MG101 and MG132, which inhibit proteasome function (11, 12), prevented the TPA-induced depletion of the \( \alpha \), \( \delta \), and \( \varepsilon \) PKC isoforms, the only TPA-responsive isoforms present in these cells (Fig. 1). E64, which shares with MG101 and MG132 the ability to inhibit calpain protease, but not the proteasome, had no effect on TPA-induced PKC depletion. We also examined the effect of these compounds on PKC \( \zeta \), a PKC isoform that is expressed in these cells but is not responsive to phorbol esters (9). As shown in Fig. 1, neither MG101 nor MG132 had any effect on PKC \( \zeta \). These data implicate the ubiquitin-proteasome pathway in the phorbol ester-induced depletion of PKC.

PKC isoforms become ubiquitinated upon TPA treatment.

The data in Fig. 1 demonstrate that compounds which inhibit proteasome function inhibit TPA-induced downregulation of PKC. Therefore, it is predicted that the affected PKC isoforms should become ubiquitinated in response to TPA. In Fig. 1, it was also observed that the anti-PKC \( \delta \) antibody recognized several higher-molecular-weight species within 30 min after TPA treatment. The appearance of these higher-molecular-weight species of PKC \( \delta \) is consistent with the rapid ubiquitination of PKC \( \delta \) in response to TPA. To investigate directly whether PKC isoforms were being ubiquitinated in response to TPA, we performed Western blot analysis of PKC isoform immunoprecipitations with antiubiquitin antibody. As shown in Fig. 2, ubiquitination of PKC \( \alpha \), \( \delta \), and \( \varepsilon \), but not PKC \( \zeta \), was detected within 30 min of TPA treatment. By 6 h, the ubiquitinated PKC isoforms were no longer detectable. However, when MG101 was used to inhibit proteasome, the ubiquitinated isoforms were still present 6 h after TPA treatment (Fig. 2). Interestingly, 24 h of treatment with MG101 alone resulted in a significant accumulation of ubiquitinated forms to a limited extent for PKC \( \alpha \) and substantially for PKC \( \varepsilon \) (Fig. 2), suggesting that ubiquitination may occur in response to physiological stimuli as well as TPA. These data demonstrate that PKC isoforms \( \alpha \), \( \delta \), and \( \varepsilon \) rapidly become ubiquitinated in response to TPA treatment and that their disappearance is blocked by inhibition of proteasome.

Degradation and ubiquitination of PKC are dependent upon PKC kinase activity.

To begin to investigate the mechanism for activation of ubiquitination and proteasome deg-
PKC activation triggers ubiquitination and degradation. In Fig. 3A, it is shown that the PKC isoforms α, δ, and ε were then immunoprecipitated (IP), and the level of ubiquitinated PKC was determined by Western blot analysis with an antiubiquitin antibody. The effect of MG101 (50 μM) on ubiquitination of untreated cells and cells treated with TPA is shown. Numbers on the left are molecular weights in thousands.

PKC is ubiquitinated and downregulated in response to DG in a proteasome- and kinase-dependent mechanism. Phorbol esters bind to PKC at the site that binds the physiological activator diacylglycerol (DG) (9). As shown in Fig. 2, the proteasome inhibitor MG101 stimulated an increase in the ubiquitinated PKC isoforms α and ε, suggesting that ubiquitination is a physiological response and not an artifact of phorbol ester treatment. We therefore wished to investigate whether ubiquitination and downregulation of PKC occur in response to DG. As shown in Fig. 5, the α and δ isoforms and to a lesser extent the ε isoform were all downregulated in response to the DG dioctoylglycerol (DiC8). This downregulation was sensitive to both proteasome and PKC inhibitors (Fig. 5A). The PKC α-specific Go6976 prevented downregulation of the α isoform specifically. We also wished to determine whether DG-stimulated ubiquitination of PKC isoforms. We added DiC8 to the 3Y1 cells and examined ubiquitination as in Fig. 2. In Fig. 5B, it is shown that DiC8 stimulated ubiquitination of PKC δ. The ubiquitination of PKC δ was inhibited by the PKC inhibitors staurosporine, bisindolylmaleimide II, and rottlerin but not by the proteasome inhibitor MG101 or the PKC inhibitor Go6976 (Fig. 5B). These data suggest that PKC isoforms become ubiquitinated and downregulated by the physiological stimulus of DG as well as by the tumor-promoting stimulus of TPA and that downregulation is dependent upon an active kinase.

TPA-induced transformation of 3Y1 cells overexpressing c-Src is blocked by proteasome inhibitors. In cells overexpressing c-Src, TPA treatment causes the appearance of transformation that is due to the depletion of PKC δ (7). We therefore investigated whether inhibitors of the ubiquitin-proteasome pathway could prevent the transformed phenotype induced by TPA in the c-Src-overexpressing cells by preventing the depletion of PKC δ. As shown in Fig. 6A, the proteasome-specific inhibitor MG101 prevented the morphological transformation of the c-Src-expressing cells induced by TPA, whereas the nonspecific protease inhibitor E64 did not prevent the morphological transformation induced by TPA. The proteasome inhibitors had no effect on the transformed phenotype induced by v-Src (Fig. 6A). The ability of MG101 to prevent the TPA-induced morphological transformation was not likely due to any effects that proteasome inhibition have upon cell cycle progression (10), since aphidicolin, which blocks cells at the G1/S boundary, did not prevent the transformed phenotype induced by TPA (Fig. 6B). These data suggest that PKC δ is ubiquitinated and downregulated in response to TPA and that downregulation is dependent upon an active kinase.

PKC was determined by Western blot analysis with an antiubiquitin antibody. The effect of MG101 (50 μM) on ubiquitination of untreated cells and cells treated with TPA is shown. Numbers on the left are molecular weights in thousands.
G1/S boundary of the cell cycle (5), had no effect on the TPA-induced morphological transformation (data not shown). In addition, MG101 had no effect on the translocation of the PKC isoforms induced by TPA (Fig. 6B). Thus, the effect observed in Fig. 6A is not due to the inability to translocate PKC isoforms to the membrane. These data suggest that PKC δ is downregulated by the ubiquitin-proteasome pathway and that this pathway is critical for the TPA-induced tumor promotion, as reported previously (7).

**DISCUSSION**

In this report, we have shown that downregulation of PKC in response to tumor-promoting phorbol esters is via the ubiquitin-proteasome pathway. In response to TPA, PKC isoforms α, δ, and ε all became ubiquitinated within 30 min and
were degraded within 6 h in 3Y1 rat fibroblasts. Proteasome inhibitors prevented TPA-induced PKC downregulation but not ubiquitination of the PKC isoforms. Ubiquitination and downregulation of PKC isoforms were dependent on an active PKC kinase. We previously demonstrated that the downregulation of PKCΔ was responsible for the tumor-promoting effects of TPA on 3Y1 cells overexpressing c-Src (7). Consistent with PKCΔ downregulation being important for the tumor-promoting effects observed previously, the proteasome inhibitor MG101, which prevented PKCΔ downregulation in response to TPA, also prevented the TPA-induced transformation of the c-Src-overexpressing cells. Thus, the data presented here implicate the ubiquitin-proteasome pathway in phorbol ester-induced tumor promotion.

Interestingly, treatment of 3Y1 cells with MG101 induced the appearance of PKC polyubiquitinated forms, especially for PKCε, which tends to be the most constitutively activated isoform in these cells (18). This suggested that ubiquitination of PKC is a physiological response and is not unique to the response to phorbol esters. Consistent with this hypothesis, ubiquitination and downregulation were observed in response to an exogenously provided DG. DG was less potent than TPA at inducing ubiquitination and downregulation of PKC; however, this was most likely because DG can be metabolically converted to other lipids such as phosphatidic acid and monoacylglycerol.

The data presented here do not demonstrate the complete mechanism of activation of the ubiquitin-proteasome pathway; however, it is apparently regulated at the level of ubiquitination. Of special interest is the requirement for the kinase activity of the PKC isoforms. Compounds that inhibit activation of PKC prevented PKC downregulation and ubiquitination in response to TPA. Additionally, a kinase-dead PKCα was completely resistant to TPA-induced downregulation. Since phorbol esters still lead to the activation and downregulation of PKC isoforms Δ and ε in cells expressing the kinase-dead PKCα, ubiquitination is apparently isoform specific and the activation of one PKC isoform does not stimulate ubiquitination and downregulation of other inactive PKC isoforms. Moreover, since the cells expressing the kinase-dead PKCα likely still express wild-type PKCα, which would be activated by TPA, it is not likely that PKCα activates a PKCα-specific ubiquitination system, because this would result in the degradation of the kinase-dead PKCα. Since the defect in the kinase-dead PKCα mutant that was not degraded in response to TPA was in the ATP-binding site, activation of the ubiquitin-conjugating system is likely stimulated by a conformational change in PKC that involves ATP binding or hydrolysis. This suggests a suicide model for regulation of PKC where upon activation, PKC becomes ubiquitinated and thereby targeted for degradation in a negative feedback control mechanism.
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FIG. 6. TPA-induced transformation of 3Y1 cells overexpressing c-Src is blocked by proteasome inhibitors. (A) 3Y1 cells overexpressing c-Src were either untreated or treated with TPA (400 nM; 10 h) in the presence of either MG101 (50 μM) or E64 (50 μM), and the morphology of the cells was examined. The effect of MG101 on v-Src-transformed 3Y1 cells is also shown. (B) The ability of TPA to induce translocation of the PKC isoforms from the cytosol to the membrane in the presence of MG101 and E64 was investigated by Western blot analysis of the PKC isoforms present in the cytosolic and membrane fractions before and after TPA treatment.

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