Increased Expression of Protein Kinase Cβ Activates ERK3*

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In a prior study, we have shown that stable transfection of expression plasmids for protein kinase Cβ1 (PKCβ1) or PKCβ2 into differentiated colon cancer cells led to elevated levels of PKCβ1 or PKCβ2 protein and PKCβ1 kinase activities in the transfectants, without altering PKCa levels. PKCγ is not found in these cells, so the major modulation was in PKCβ. PKCβ transfector cells exhibited blocked differentiation, increased growth rate in athymic mice, and restoration of the basic fibroblast growth factor response pathway. In this study, we have extended the analysis of these PKCβ transfectants to the mitogen-activated protein kinase ERK3. Analysis of cell lysates on the mitogen-activated protein kinase substrate myelin basic protein by in gel kinase assay showed increased activity at 63 kDa, the size of ERK3, in each of two PKCβ1 and each of two PKCβ2 transfectants compared with the vector control transfector. ERK3 was expressed at equal abundance in PKCβ1, PKCβ2, and control transfector cells as demonstrated by Western blotting and by immunoprecipitation with anti-ERK3 monoclonal antibody. However, a >10-fold increase in ERK3 activity in each PKCβ transfector was shown by immunoprecipitation with anti-ERK3 monoclonal antibody followed by either immune complex kinase assay or by in gel kinase assay. Thus, while overexpression of transfected PKCβ does not lead to overexpression of ERK3, it does lead to constitutive activation of ERK3. A causal link between PKCβ overexpression and ERK3 activation was established because 12-O-tetradecanoylphorbol-13-acetate treatment down-regulated both PKC and ERK3 activities in both PKCβ1 transfectants. ERK3 activity was found in nuclear and membrane fractions in each PKCβ transfectant, in contrast to controls, perhaps accounting for constitutive activation of ERK3 in cells with elevated levels of PKCβ1 or PKCβ2.

In previous studies, we found that differentiation of colon carcinoma cells down-regulated the abundance and activity of PKCβ1 while not altering levels of other PKC isozymes present in these cells. Each of two independently cloned colon carcinoma lines that display differentiation characteristics of mature fluid transporting colon enterocytes down-regulated PKCβ1 activity and abundance 5–10-fold compared with each of two undifferentiated lines (1). Neither differentiated line was capable of transmitting mitogenic signals from basic fibroblast growth factor to p57MAPK (1). Both differentiated and undifferentiated lines maintained equal levels of the PKC isozymes α, ε, and γ, and none of the four lines exhibited either PKCγ or PKCδ (1), limiting at least the major, if not the entire, PKC modulation to the PKCβ isozyme. We transfected both splice variants of PKCβ (2), PKCβ1 and PKCβ2, into one of these differentiated colon carcinoma lines, HD3. The HD3 transfectant cells with increased PKCβ1 and PKCβ2 expression but unaltered PKCa levels were blocked in differentiation, had constitutively activated both the ERK1-related p57MAPK and ERK1, restored the basic fibroblast growth factor response pathway, and had acquired the capacity for rapid growth in athymic mice (3).

In this study, we have extended our studies of PKCβ transfectants to the MAP kinase ERK3. ERK3 is structurally related to the more well studied ERK1 and ERK2, with a 43% overall homology (4), but little is known of the function or activation of ERK3. Two human homologues of the rat erk3 gene have been cloned, one of 63 kDa with a 73% amino acid identity (5) and, more recently, a 97-kDa homologue that has a 98% homology to rat Erk3 through the first 500 amino acids followed by a unique 178-amino acid extension (6). 97-kDa ERK3 has been shown to have kinase activity on histone H1 when fibroblasts are stimulated with serum or phorbol ester but not insulin, insulin-like growth factor 1, or epidermal growth factor (6).

ERK1 and ERK2 are activated by phosphorylation on tyrosine and threonine in a TEF site in subdomain VII (7–10) by a well known pathway through MEK and MEK kinase (summarized in Ref. 11). In contrast, ERK3 has an SEG site at the homologous site in subdomain VII (4) and may be activated in vivo through different MEKs and MEK kinases or other kinases. We have asked in this study whether elevated levels of PKCβ1 and PKCβ2, which block differentiation in colon cancer cells (3), alter the activation or abundance of ERK3.

EXPERIMENTAL PROCEDURES

Materials—[125I]-Protein A and [γ-32P]ATP were obtained from DuPont NEN; myelin basic protein and TPA were from Sigma; protein A-Sepharose was from Pharmacia Biotech Inc; and Immobilon-P polyvinylidene difluoride transfer paper was from Millipore Corp. Anti-pan-ERK M antisera and anti-ERK3 monoclonal antibody were purchased from Transduction Laboratories, and sheep anti-mouse IgM was from BIODESIGN International.

Cell Culture—Cells were maintained in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum as described (12). Partial Purification of PKC from PKCβ Transfectants—The protocol was essentially that used before (1). Briefly, log-phase cultures from each line were washed twice in phosphate-buffered saline, swelled for 15 min on ice, and then homogenized in hypotonic buffer B (10 mM Hepes (pH 7.5) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, 5 mM EGTA, 2 mM EDTA, and 2 mM dithiothreitol). A membrane pellet was prepared by centrifugation of the homogenate at 100,000 x g for 1 h at 4 °C. The supernatant was used to prepare the cytosolic PKC preparation, which was absorbed to DE52 columns and eluted in 0.1 M NaCl after washing, exactly as

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1 The abbreviations used are: PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, mitogen-activated kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; MBP, myelin basic protein; mAb, monoclonal antibody.
described (1). The membrane pellet was extracted with 0.5% Triton X-100 in buffer B for 30 min on ice and pelleted at 10,000 × g for 10 min to remove debris. The supernatant was the membrane extract.

Kinase Assays in MBP-containing Polycrylamide Gels—The method was adapted from one previously used (1). Log-phase cells were lysed in buffer A (20 mM Tris-HCl (pH 7.5) containing 10 μg/ml aprotinin, 20 μg/ml leupeptin, 24 μg/ml phenylmethylsulfonyl fluoride, 1 mM EGTA, 2 mM EDTA, 1 mM NaF, 100 μM Na3VO4, and 2 mM benzamide), boiled in Laemmli sample buffer for 2 min, and then electrophoresed in a 7.5% SDS-polyacrylamide gel (0.5 mm × 5 cm) containing 0.5 mg/ml MBP. After fixation with 20% 2-propanol in 50 mM Tris-HCl buffer (pH 8.0) for 2 h, SDS was removed by washing the gel for 2 h in several gel volumes of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, with frequent changes. The MBP kinases were then redenatured with 6 M guanidine HCl for 2 h and then renatured by 10 washes of 20 min each in several gel volumes of 50 mM Tris-HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol, with frequent changes. The MBP kinases were then incubated on ice for 20 min to allow swelling. The cells were then homogenized in a Dounce homogenizer (20 strokes), and the homogenate was incubated at 4 °C for 2 h. The supernatant was the membrane extract.

Cell Fractionation—Cells were homogenized in buffer A containing protease and phosphatase inhibitors, and a cytosol fraction, and the pellet was solubilized in hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspension was incubated on ice for 20 min to allow swelling. The cells were then homogenized in a Dounce homogenizer (20 strokes), and the homogenate was loaded onto 1 ml of 1 n sucrose in lysis buffer and centrifuged at 10,000 × g for 10 min to pellet nuclei. The supernatant above the sucrose cushion was centrifuged at 150,000 × g for 30 min to prepare the cytosol fraction, and the pellet was solubilized in hypotonic lysis buffer containing 0.5% Nonidet P-40 and 0.1% sodium deoxycholate and centrifuged at 10,000 × g for 5 min to remove insoluble material. Nonidet P-40 and sodium deoxycholate were added to the cytosol fraction to give equal concentrations of these detergents in all fractions.

RESULTS

ERK3 Activated in PKCβ1 and PKCβ2 Transfectants—In our earlier study (3), four PKCβ transfectants (PKCβ1-1, PKCβ1-2, PKCβ2-1, and PKCβ2-2) were isolated that exhibited elevated activity and abundance of PKCβ1 and PKCβ2, respectively, compared with the vector control transfected. PKCα levels were not altered, and PKCγ was not expressed in these transfectants. Lysates of both PKCβ1 and both PKCβ2 transfectants and one empty vector transfected line were analyzed for activation of MAP kinases by an in gel kinase assay with immobilized MBP as the substrate. A major band of activity was seen at roughly 63 kDa in each PKCβ transfecant, but not in the control cells (Fig. 1), suggesting either activation of a kinase or increased abundance of a kinase. Longer exposure of the gel was necessary to detect p59MAPK or p44MAPK/ERK1 (Ref. 3 and data not shown). ERK3 was immunoprecipitated from both PKCβ and control transfected lines with monoclonal antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-ERK3 and demonstrated that each PKCβ transfectant and the control transf ectant expressed equal amounts of the p63 form of ERK3 (Fig. 2). Larger molecular mass forms of ERK3 (6) were not detected in these cells. Western blotting of total cell lysates with mAB to ERK3 also demonstrated equal abundances of ERK3 in PKCβ and control transfected cells (Fig. 3), as did a similar experiment with a pan-ERK antiserum (data not shown). Immunoprecipitation with a pan-ERK antiserum from 35S-prelabeled cells also demonstrated equal abundance of a 63-kDa MAP kinase in PKCβ1 and control transfected cells (3). Thus, overexpression of PKCβ1 or PKCβ2 did not lead to overexpression of ERK3.

ERK3 was immunoprecipitated from each PKCβ and control transfecant, and ERK3 activation was then analyzed in two ways. An immune complex kinase experiment was performed using MBP as the substrate. Both PKCβ1 and both PKCβ2 transfectant lines exhibited 13-fold more ERK3 activity than the vector control in triplicate assays (Fig. 4). ERK3 immunoprecipitates were also analyzed by in gel kinase assay on immobilized MBP. This assay allows one to visualize the kinase reaction product at the molecular mass of the kinase, confirming the identity of the MAP kinase immunoprecipitated by anti-ERK3 mAb as the p63 form of ERK3 (Fig. 5). Kinase product was only visualized in immunoprecipitates from the PKCβ transfectant lines and was at least 10-fold control levels. Similar activation of ERK3 in PKCβ transfectants was also observed using the pan-ERK antiserum in two additional immunoprecipitation experiments (data not shown). Therefore, these experiments show that increased levels of PKCβ1 and PKCβ2 correlate in each of four transfected lines with activation of ERK3, but not with an increase in ERK3 abundance.

Down-regulation of PKC Levels by TPA Treatment Decreases ERK3 Activity—Both PKCβ1 transfectant lines and the vector control were treated for 24 h with 100 ng/ml TPA or the diluent, 0.1% dimethyl sulfoxide. PKCβ was partially purified by DE52 chromatography from the cytosols of each treated line (see “Experimental Procedures”). Total calcium/phosphatidylycerine-dependent PKC activities consisted of the β1, β2, and α-
isoforms as γ is not present. A 20% decrease in total calcium/phosphatidylserine-dependent activities (data not shown) was induced by 24 h of TPA treatment and was not further decreased by 48 h of TPA treatment. Down-regulation of PKC activity decreased the p63MBPK activity in both PKCβ1 transfectants (Fig. 6). To confirm that the TPA-down-regulated p63MBPK activity was ERK3, ERK3 was immunoprecipitated from TPA- and diluent-treated PKCβ1 transfectant cells, and an immune complex kinase assay was performed, using MBP as the substrate. Decreased ERK3 activity was seen in TPA-treated PKCβ1 cells compared with diluent-treated control cells (4299 ± 34 cpm versus 5341 ± 53 cpm, triplicate assays). Thus, the decrease in p63MBPK seen in TPA-treated PKCβ1 transfectants (Fig. 6) was correlated with a decrease in ERK3 activity immunoprecipitated with specific mAB, and both decreases were roughly 20%.

It has been reported that PKCβ2 found in colonocytes is not down-regulated efficiently by TPA, in contrast to other PKC isoforms (14). We were not able to decrease p63MBPK activity, as measured by in gel kinase assay, or ERK3 activity, as assayed by immune complex kinase assay after immunoprecipitation, by TPA treatment of PKCβ2 transfectant cells. Since neither activity was affected, however, this experiment again correlated ERK3 activity with p63MBPK activity (data not shown).

Constitutive Activation of ERK3 in Each PKCβ Transfectant Line Correlates with Nuclear Localization—Other investigators have shown that treatment of serum-deprived HeLa cells with serum or TPA led to activation of p44MAPK/ERK1 as shown by increased tyrosine and threonine phosphorylation of p44MAPK and translocation of the activated p44MAPK to the nucleus (13). Since we had established that the p63MBPK activity seen in cell lysates from PKCβ transfectants was ERK3, we used the simple, quantitative in gel kinase assay to determine whether constitutively activated ERK3 in PKCβ transfectants would be found in the nucleus without growth factor treatment. Protein from cytosolic, nuclear, and membrane fractions was assayed proportionally to the total amount of protein in each fraction. Each of the four PKCβ transfectant lines exhibited more ERK3 activity in the nuclear fractions than was seen in the vector control in duplicate experiments, on immobilized MBP by the in gel kinase reaction (Fig. 7). The PKCβ transfectants constitutively displayed a characteristic of mitogen-treated control cells since treatment of vector control cells with a mitogenic concentration of epidermal growth factor caused an appearance of p63MBPK/ERK3 activity in the nuclear fraction (data not shown). ERK3 and two larger, uncharacterized MBP kinases were also present in membrane fractions from each of the four PKCβ transfectant lines (Fig. 8). In these experiments, only membrane and nuclear fractions, not cytosols, were analyzed proportionally to the total amount of protein in each fraction, so larger amounts of proteins from these fractions...
Activated ERK3 kinase.

The abundance and therefore the activities of PKC could be size-fractionated. Treatment of the parental HD3 cells with growth-inhibiting concentrations of transforming growth factor β1 decreased the activity of the larger MBP kinases found in the membrane fraction in Fig. 8, linking modulation of these activities with growth modulation (15). Therefore, elevating the abundance and therefore the activities of PKCβ1 and PKCβ2 by transfection led to activation of several kinases including ERK3, several of which may play roles in growth control.

Constitutive activation of ERK3 might be related to the enhanced levels of ERK3 found in nuclear and membrane fractions of PKCβ transfectant cells. The nuclear and membrane locations of some of the ERK3 activity makes it unlikely that the MBP kinase activity immunoprecipitated with both ERK3-specific mAb and pan-ERK antisera (Figs. 4 and 5) was a 63-kDa proteolytic fragment of PKCβ contaminating both immunoprecipitations.

**DISCUSSION**

One of properties of the p44/p42MAPK isoforms is their capacity for activation by the phorbol ester class of PKC activators (11, 16). We have shown here and in an earlier study (3) that overexpression of one PKC isoform, PKCβ2, in colon cancer cells blocks differentiation and activates not only the well studied MAP kinase ERK1, but also ERK2 and the ERK1-related p57MAPK. Thus, permutation in only one PKC isozyme, PKCβ2, activates several MAP kinases, each of which may have different sites of action, either within the nucleus to activate different sets of transcription factors or at the cytoskeleton to phosphorylate microtubule-associated proteins, destabilizing microtubules and thus cytoskeletal structure and organization (11, 16). Several MAP kinases have been isolated from various species from Drosophila to Xenopus to human, and these can be divided into different families depending on DNA sequence homologies. MAP kinase families are characterized by high homology between family members; for example, ERK1 exhibits 90% sequence homology to ERK2 (4). However, these “classical” MAP kinases exhibit much lower sequence homology to other MAP kinases. ERK1 has 43% homology to the ERK3 family (4); 40% homology to JUN kinases and p38MAPK, which are activated by cellular stress such as protein synthesis inhibitors, osmotic shock, or UV light (11, 17, 18); and 40% homology to ERK5 (19), a newly cloned, larger MAP kinase. These different MAP kinase families are expressed preferentially in various cell types, suggesting that they may have different roles in proliferation, differentiation, or embryonic development. Little is known about the activation or role in cell proliferation or differentiation of any of the ERK3 family members. 97-kDa ERK3 has been shown to have kinase activity on histone H1 when fibroblasts are stimulated with serum or phorbol ester, but not insulin, insulin-like growth factor 1, or epidermal growth factor (6). ERK1 and ERK2 and the JUN kinases are activated by phosphorylation on tyrosine and threonine on a TXXY site in subdomain VII (7–11, 17, 18) by a well known pathway through specific MEKs and MEK kinases (11). In contrast, ERK3 has an SEG site at the homologous site in subdomain VII (4) and may be activated by other kinases in vivo.

There are at least 12 PKC members classified into three groups: Ca2+-dependent, phosphatidylserine- and diacylglycerol-dependent conventional PKC isozymes (α, β, γ, and δ); Ca2+-independent novel PKC isozymes (ζ, ε, μ, η, and ι); and Ca2+- and diacylglycerol-independent, phosphatidylserine-dependent atypical PKC isozymes (ζ, λ, and ζ). Roles for PKC isozymes may depend on cell type, and the ratios of the different PKC isozymes within a cell also may alter a cell’s response to PKC activators. Craven and DeRubertis (20) have found that treatment of rats with a colon carcinogen led to a relative increase in PKCβ expression, together with a decrease in PKCα expression in colon epithelial cells, leading to an increased PKCβ/PKCα ratio. Marian and co-workers (21) have measured PKC isoform protein levels in normal human colonocytes, human benign colon tumors, and human malignant colon tumors and found a decrease in PKC isoform levels in both benign and malignant tumor cells, with much less decrease in PKCβ, leading to an enrichment of PKCβ levels in colon tumors relative to other isoforms detectable in colon tumor tissue: α, ε, δ, ζ, and η. The benign tumor cells with increased PKCβ/PKCα ratio were responsive to TPA, a mitogen for colon adenoma cells (21, 22). The loss in most PKC isoforms on the protein level in colon tumor tissue is consistent with earlier reports that colon tumors express less total PKC mRNA than normal tissue (23) and, more recently, less PKCβ and less PKCα mRNAs (24).

We found in earlier studies that differentiation of colon carcinoma cells to fluid-transporting enteroctylic-like cells led to a 5-10-fold decrease in PKCβ abundance and activity, with no change in abundance of the other PKC isoforms detectable, α, ε, and ζ (1). These studies, like the studies cited above, point to a role for enhanced levels of PKCβ relative to the other isoforms in colon tumor progression. Transfection of either PKCβ isoform, PKCβ1 or PKCβ2, into such differentiated colon carcinoma cells restored the undifferentiated phenotype and proliferative response to basic fibroblast growth factor and allowed more rapid growth in athymic mice (3). The mechanism, at least in part, for this blocked differentiation was a constitutive activation of both ERK1 and the ERK1-related p57MAPK (3). In the current study, a third MAP kinase, ERK3, was found also to be constitutively activated by overexpression of PKCβ1 or PKCβ2. ERK3 was found associated with the nucleus and membrane in PKCβ transfectant cells. Nuclear location may allow the activated ERK3 to act as a transcription factor kinase, while membrane location may allow association with the microtubule cytoskeleton. ERK1 and ERK2 are associated with the microtubule cytoskeleton in NIH 3T3 fibroblasts (25). MAP kinases are known to phosphorylate microtubule-associated proteins, causing microtubule instability (11), per...
haploinsufficiency leading to the cellular and nuclear shape changes that occur during S phase and mitosis.

Our studies have correlated increased expression of PKCβ1 and PKCβ2 with increased proliferation in colon cancer due to activation of multiple MAP kinases. Others have found constitutive activation of MAP kinases in human renal cancers (26). In a series of 25 cases of paired normal kidney tissue and renal tumors, constitutive activation of MAP kinases, as determined by the appearance of phosphorylated forms of ERK2, was found in 48% of the cases (26). Others have found PKCβ to be elevated in activity in invasive gastric cancers (27). PKCβ2 is required for the proliferation of K562 human erythroleukemia cells (28). Overexpression of PKCβ2 in HL-60 promyelocytic leukemic cells enhances their proliferation and makes them resistant to TPA-induced differentiation (29). PKCβ2 translocates to the nuclear envelope and phosphorylates lamin B in cells treated with mitogenic stimuli, but not with differentiation inducers like TPA (29). Thus, proliferation of HL-60 cells is correlated with nuclear translocation of PKCβ2 and its phosphorylation of nuclear envelope lamin B, part of the nuclear membrane breakdown that occurs when cells enter mitosis. It would be of interest to determine whether nuclear forms of ERK3 could be responsible for this phosphorylation. Thus, overexpression of PKCβ2 has, in several cell types, been associated with increased proliferation and blocked differentiation.

The role of PKCβ1 is more problematic. Other investigators have shown that expression of elevated levels of PKCβ1 in uncloned HT29 colon carcinoma cells (30) and SW480 colon carcinoma cells (31) inhibited tumorigenicity and cell growth. The uncloned HT29 line and the SW480 line are both undifferentiated and highly tumorigenic in our studies. PKCβ1 was overexpressed following stable transfection of a poorly tumorigenic, differentiated cell line with low PKCβ levels (1), and the transfectants simply had restored PKCβ1 levels to those characteristic of undifferentiated parental cells (3), not significantly above this level as in the other cited studies. In this and earlier studies, we have shown that at least part of the mechanism of action of PKCβ in colon cancer cells is constitutive activation of multiple MAP kinases. It will now be necessary to determine the spectrum of substrates for the ERK1-like p52MAPK, ERK3, and ERK1 in colon carcinoma cells in both the nucleus and cytoskeleton. In this way, it may be possible to identify the proximal effectors of PKCβ.

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