Sustained activation of protein kinase C downregulates nuclear factor-κB signaling by dissociation of IKK-γ and Hsp90 complex in human colonic epithelial cells

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Activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) triggers cellular signals that lead to the activation of the transcription factor NF-κB (nuclear factor kappaB) in various cell types. In addition to NF-κB activation by short-time PMA treatment, here we report that the prolonged exposure of human colonic cancer epithelial cells treated with PMA can also lead to a persistent inhibition of NF-κB activation. PMA selectively causes the degradation of 1κB kinases (IKKs) including IKK-γ and IKK-β, and subsequent inhibition of tumor necrosis factor (TNF) induced IKK and NF-κB activation in human colon cancer cell line HCT-116, but not in other gastrointestinal tract cells. The use of Ro-318220 and GO-6983, general PKC inhibitors as well as MG-132, a proteasome-specific inhibitor, abrogated PMA-induced degradation of IKK-γ and recovered the activation of IKK by TNF, suggesting that IKK complex is predominantly degraded by the proteasome pathway in a PKC-dependent manner. We also found that IKK-γ strongly associates with heat shock protein 90 (Hsp90) in HCT-116 cells, and that this interaction was dramatically reduced after exposure to PMA. Furthermore, high levels of Hsp90 expression and enhanced association with IKK were observed in human colon cancer tissues. Taken together, these results suggest that long-term activation of PKC by PMA inhibits NF-κB system in case of colon cancer cells by disrupting the interaction of IKK-γ with Hsp90, which may represent a novel regulatory mechanism of PKC-dependent cellular differentiation and limited proliferation of colonic epithelial cells.

Introduction

The transcription factor NF-κB (nuclear factor kappaB) is critical in a number of cell functions, including growth, survival, and inflammatory and immune responses (1–5).

Abbreviations: GST, glutathione-S-transferase; Hsp90, heat shock protein 90; IHC, immunohistochemistry; INK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappaB; NIK, NF-κB-inducing kinase; PKC, protein kinase C; PMAC, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor.

Deregulated activity of NF-κB pathway has also been observed and linked to the progression of several human malignancies (6–8). Since it is becoming increasingly clear that NF-κB can modulate the aspect of cell growth and death, understanding the regulation of NF-κB activity is likely to shed insight into cancer progression or treatment.

In most types of cells, inactive NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory proteins known as IκBs (9,10). Upon cell activation by various stimuli, including pro-inflammatory cytokines TNF (tumor necrosis factor) and IL-1, IκBs are phosphorylated at residues 32 and 36, which trigger the ubiquitination and subsequent degradation of IκBs through the proteasome. The degradation of IκBs leads to the release of NF-κB and allows its translocation into the nucleus and subsequent activation of a number of target genes (2). The protein kinase responsible for the stimulus-dependent phosphorylation and degradation of IκBs is a heterodimer of three subunits, consisting of at least two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ (also known an NEMO, NF-κB essential modulator) (10–12). While IKK-α and IKK-β are absolutely essential for IκB phosphorylation and NF-κB activation, IKK-γ forms a tetrameric scaffold that can assemble two kinase dimers to facilitate transautophosphorylation (13,14). Although IKK-γ is required for kinase activity by the complex, it is not itself a kinase; rather, IKK-γ appears to mediate crucial protein–protein interactions, possibly with upstream activators of the kinase complex (15). Recently, heat shock protein 90 (Hsp90) and Cdc37 have been found to associate stoichiometrically with the IKK complex, which may contribute to the stabilization, activation and/or shuttling of IKKs to the plasma membrane, because Hsp90 regulates stability and function of a unique complement of signaling molecules (16–19).

Phorbol esters such as phorbol 12-myristate-13-acetate (PMA) activate classic (α, β I, β II, γ) and novel (δ, e, θ) protein kinase C (PKC) isoforms by binding to diacylglycerol (DAG) receptor site in the N-terminus of these proteins, inducing their translocation to their sites of action (20,21). The PKC family has long been known to play pivotal roles in controlling cell growth and differentiation by regulating the activity of transcription factors such as AP-1 and NF-κB, and their biological effects exerted by the activation of PKC with phorbol esters may be cell- or tissue-specific (22–24). An earlier study showed that PMA can induce the rapid and transient activation of NF-κB through the novel IKK complex, IKKe and the 90 kDa ribosomal S6 kinase (p90(rk)) that involves the cellular proliferation (25). However, several studies have demonstrated that sustained activation of PKC family members with PMA caused growth inhibition and decreased tumorigenicity by inducing cellular differentiation in several colon cancer cell lines (26–28). These suggest that diversified functions of PKC in the regulation of cellular proliferation and differentiation depend on the duration of...
stimuli. Overexpression of PKC has been reported to associate with inhibition of proliferation, cell cycle arrest, enhanced differentiation and accelerated apoptosis in several colonic cell lines (29–31). Consistent with this potential cellular differentiation role for PMA, significantly decreased protein expression and/or total PKC biochemical activity were found in experimental model of colon cancer as well as human colon cancer tissue (32,33).

Although it is well established that sustained activation of PKC mainly acts as involvement of colonic epithelial cell differentiation, there is no report on the alteration of NF-κB signaling pathway during PMA-induced epithelial differentiation. In this study, we made an unexpected observation that activation of PKC by PMA downregulates NF-κB signaling pathway, followed by degradation of IKK-γ and IKK-β in several colonic epithelial cells, which distinguishes the cells from other gastrointestinal tract cell lineages. More interestingly, IKK-γ was strongly associated with Hsp90, and this interaction was inhibited by PMA treatment, leading to suppression of NF-κB in these cells.

Materials and methods

Reagents

Glutathione S-transferase (GST)-c-jun (residues 1–79) and GST-IκB-α (residues 1–54) were expressed and purified from Escherichia coli as described previously (34). Anti-JNK-1 antibody was purchased from BD Pharmaning (San Diego, CA, USA). Anti-IκB-α, anti-IκB-α, anti-IκB-α, anti-IKK-α, anti-IKK-α, anti-NIK (NF-κB-inducing kinase), anti-Hsp27, anti-ubiquitin and anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Hsp70 and anti-Hsp90 were purchased from Stressgen (Ann Arbor, MI, USA). Anti-β-actin and anti-flag antibodies, cyclodexhime (CHX) and ammonium chloride were purchased from Sigma (St. Louis, MO, USA). Peroxidase-conjugated secondary antibodies were purchased from Calbiochem (Darmstadt, Germany). Recombinant mouse TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Protein A- and G-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture and transfection

HEK-293 cells, human colonic epithelial cells [HCT-116 (American Type Culture Collection, Bethesda, MD, USA) (ATCC, CCL-247, SW-480 (ATCC, CCL-220), HMT (kindly donated by Dr Young S.Kim; Gastrointestinal Research Lab, University of California, San Francisco, CA, USA) and LS174T (ATCC, CL-188)], human hepatocellular carcinoma cells (Hep 3B, ATCC, HB-8064), human gastric carcinoma cells (SNU-1, ATCC, CRL-5971), human pancreatic adenocarcinoma cells [SW-1990 (ATCC, CRL-2172) and Capan-2 (ATCC, HTB-80)], were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfection experiments in HCT-116 cells and HEK-293 cells were performed with Lipofectamine PLUS reagent by following the instruction provided by the manufacturer (GIBCO BRL; Carlsbad, CA, USA).

Western blot analysis

After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris at pH 7.6, 0.5% NP-40, 250 mM NaCl. 3 mM EDTA, 2 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 10 mM N-ethylmaleimide), and lysates were incubated with anti-IκB-α antibody and protein G-Sepharose. All immunoprecipitates were washed four times with lysis buffer, boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 8% polyacrylamide gel. Immunocomplex kinase assay for IKK and c-Jun N-terminal kinase (JNK) were performed as described previously (34). In brief, whole cell extracts were immunoprecipitated with the anti-IκB-α and anti-JNK1 antibodies, respectively, and protein A-Sepharose beads. The beads were washed with lysis buffer, and then kinase assay was performed in complete kinase assay buffer (20 mM HEPES at pH 7.5, 20 mM β-glycerol phosphate, 10 mM MgCl2, 1 mM DTT, 10 mM NP40, 50 μM sodium vanadate, 20 μM ATP) with the addition of γ-[32P]ATP and 1 μg of GST-IκB-α and GST-c-Jun (1–79), respectively, as substrate. After 20 min at 30°C, sample buffer was added and proteins were resolved in 4–20% SDS–polyacrylamide gels, and phosphorylated substrates were visualized by autoradiography.

 Luciferase assay

HCT-116 cells were cotransfected with p2xNF-xB-Luc, pRSV-β-galactosidase and HA-tagged IκB-α, IκB-β, IκK-γ and NIK constructs as indicated in the figure legends. Twenty-four hours after transfection, cells were treated with TNF (15 ng/ml) or PMA (10 nM) for additional 10 h, and luciferase activities in these cells were measured using a luciferase assay kit (Promega; Madison, WI, USA). Luciferase activity was normalized relative to β-galactosidase activity of each sample.

Immunohistochemistry

Expression of Hsp90 was analyzed by immunohistochemistry (IHC) on paraffin-embedded tissue sections from eight colon cancer tissues. Three μm thick sections from the paraffin blocks were used for IHC with mouse EnVision-HRP detection system (Dako, Carpenteria, CA, USA). The monoclonal mouse antibody for Hsp90 (Ann Arbor, MI, USA) was used for IHC. After deparaffinization and antigen retrieval by pressure cooker in 10 mM sodium citrate buffer (pH 6.0) at full power for 4 min, tissue sections were treated with 3% hydrogen peroxide for 10 min. The primary antibody was diluted (1 : 50) with background reducing diluent (Dako) and incubated for 30 min. Slides were then incubated with the EnVision reagent for 30 min. The slides were then sequentially incubated with DAB chromogen for 5 min, counterstained with Mayer’s hematoxylin and mounted. Careful rinses with several changes of TBS-0.3% Tween buffer were performed in each step. Negative control was used using mouse IgG1 isotype control and excluding the primary antibody. Cytoplasmic staining was considered as positive cells.

 Determination of cell death

After treatment, as described in the figure legends, cells were trypsinized and collected. Each sample was stained with trypan blue (Bio-Whittaker; Rockland, ME, USA) and counted with a hemacytometer. The stained cells (blue) were counted as dead cells and were expressed as a percentage of total cells. For each treatment, duplicate experiments were repeated three times.

Results

Sustained activation of PKC with PMA inhibits NF-κB activation through IKK-β and IKK-γ degradation in human colon epithelial cells, but not in other gastrointestinal tract cells

Although it is well known that PMA induces the transient activation of NF-κB in many types of mammalian cells (22,25), long-term activation effect of PKC that leads to epithelial differentiation on the NF-κB signaling is largely unknown. To characterize the NF-κB signaling pathway in human colonic epithelial cells stimulated by PMA, we compared the kinetics of the degradation and phosphorylation of IκB-α protein at various time points after TNF or PMA treatment in HCT-116 cells. As shown in the left panel of Figure 1A, when cells were treated with TNF, the degradation of IκB-α protein started to be detected at 15 min after

The lysates were mixed and precipitated with the relevant antibody and protein G-Sepharose beads by incubation at 4°C for 4 h to overnight.

For the detection of modified IκK-γ protein, HCT-116 cells were lysed in a lysis buffer (20 mM Tris at pH 7.6, 0.5% NP-40, 150 mM NaCl, 3 mM EDTA, 2 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 10 mM N-ethylmaleimide), and lysates were incubated with anti-IκK-γ antibody and protein G-Sepharose. All immunoprecipitates were washed four times with lysis buffer, boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 8% polyacrylamide gel. Immunocomplex kinase assay for IKK and c-Jun N-terminal kinase (JNK) were performed as described previously (34). In brief, whole cell extracts were immunoprecipitated with the anti-IκK-α and anti-JNK1 antibodies, respectively, and protein A-Sepharose beads. The beads were washed with lysis buffer, and then kinase assay was performed in complete kinase assay buffer (20 mM HEPES at pH 7.5, 20 mM β-glycerol phosphate, 10 mM MgCl2, 1 mM DTT, 10 mM NP40, 50 μM sodium vanadate, 20 μM ATP) with the addition of γ-[32P]ATP and 1 μg of GST-IκB-α and GST-c-Jun (1–79), respectively, as substrate. After 20 min at 30°C, sample buffer was added and proteins were resolved in 4–20% SDS–polyacrylamide gels, and phosphorylated substrates were visualized by autoradiography.

Luciferase assay

HCT-116 cells were co-transfected with p2xNF-xB-Luc, pRSV-β-galactosidase and HA-tagged IκK-α, IκK-β, IκK-γ and NIK constructs as indicated in the figure legends. Twenty-four hours after transfection, cells were treated with TNF (15 ng/ml) or PMA (10 nM) for additional 10 h, and luciferase activities in these cells were measured using a luciferase assay kit (Promega; Madison, WI, USA). Luciferase activity was normalized relative to β-galactosidase activity of each sample.
While the expression of NF-κB-inducing kinase (NIK), IKK-α and β-actin changed little, the levels IKK-β and IKK-γ were dramatically decreased at 3 h after PMA treatment in HCT-116 cells (Figure 1C). On the basis of the ability of PMA to induce IKK protein degradation, we examined the possibility that inhibition of PKC might reverse PMA response. Pretreatment of HCT-116 cells with PKC-specific inhibitors such as Ro-31-8220, Go-6983 and bisindolylmaleimide I (Bis I) completely prevented the PMA-induced IKK protein degradation (Figure 1D), indicating that the degradation of IKKs after long-term treatment of PMA is accomplished in a PKC-dependent mode in these cells.

Because PMA treatment dramatically decreased the protein expression of IKK-β and IKK-γ, but not of IKK-α and NIK, in HCT-116 cells (Figure 1C), we tested the possibility that PMA might specifically affect NF-κB activation induced by these upstream kinases. To do this, HCT-116 cells were cotransfected with a NF-κB-response reporter gene and expression constructs for IKK-α, IKK-β, IKK-γ and NIK. Overexpression of IKK-α, IKK-β and NIK, but not IKK-γ, induced activation of NF-κB reporter gene as expected (13,14) (Figure 1E). In addition, IKK-β-induced NF-κB activation was decreased by PMA treatment, whereas IKK-α- or NIK-induced NF-κB activation was little affected. These results provide further evidence that the degradation of IKK-β after long-term treatment of PMA leads to downregulation of NF-κB activity in HCT-116 cells. To study the mechanism of PMA-induced IKK-β and IKK-γ degradation, we examined the ability of several protease inhibitors to block IKK-β and IKK-γ degradation in the presence of PMA. The proteasome inhibitor MG-132 suppressed PMA-stimulated IKK-β and IKK-γ degradation, whereas the lysosome inhibitor ammonium chloride and the caspase inhibitors, benzoyloxycarbonyl-Val-Ala-Asp (ZVAD) and z-DEVD-FMK, were unable to protect the degradation of IKK-β and IKK-γ (Figure 2A and B). These results suggest that sustained activation of PKC with PMA inhibits NF-κB activation through the proteasome-dependent degradation of IKK-β and IKK-γ. To investigate whether IKK is ubiquitinated during PMA treatment, we immunoprecipitated the
endogenous IKK-γ protein from HCT-116 cells that were transfection with HA-tagged ubiquitin following stimulation with MG-132 or PMA, and analyzed by western blotting with anti-HA and anti-IKK-γ antibodies. As shown in the top panel of Figure 2C, IKK-γ-Ub conjugates were induced by either PMA or proteasome inhibitor MG-132 treatment. Furthermore, the PMA-induced ubiquitination of IKK-γ was significantly enhanced when cells were incubated with MG-132 (middle panel of Figure 2D), which indicates that the PMA-induced ubiquitination of IKK-γ may have a role in protein stability. It has been suggested that the fate of a protein that is modified by ubiquitination is determined by the type of ubiquitin linkage. Lys48-linked polyubiquitination generally targets proteins for degradation in the proteasome, whereas Lys63-linked ubiquitination can regulate protein function or protein–protein interaction (35). Given our findings that ubiquitinated IKK-γ is targeted for degradation, we examined whether PMA treatment catalyzes the formation of unique polyubiquitin chain linked through Lys48 of ubiquitin. When HA-tagged ubiquitin or single lysine ubiquitin mutants (K48R and K63R) were co-expressed with flag-tagged IKK-γ in HCT-116 cells, a point mutation at position 48 from lysine to arginine (K48R) significantly attenuated the ability of PMA-induced ubiquitination of IKK-γ relative to wild-type Ub or K63R mutant (middle panel of Figure 2D). These results provide evidences that IKK-γ is conjugated by Lys-48, but not Lys-63 polyubiquitin chain, for targeting to proteasome in response to PMA treatment in colonic epithelial cells. In contrast to the results with PMA treatment, TNF did not promote ubiquitination of IKK-γ by K63R-Ub compared with wild-type Ub or K48R-Ub (bottom panel of Figure 4D) as consistent with a previous report (36).

We next examined whether these effects in response to PMA treatment are replicable in other colonic cancer epithelial cells. In a panel of four human colonic cancer epithelial cells, treatment with PMA significantly induced IKK-γ degradation in HCT-116, SW 480, HM 7 and LSG cells (Figure 3A). In contrast, in cells from other gastrointestinal tract lineage (Hep 3B, hepatocellular carcinoma; SNU-1, gastric carcinoma; SW 1990 and Capan-2, pancreatic adenocarcinoma), PMA did not induce IKK-γ degradation (Figure 3B), suggesting that the effect of PMA is specific in the colonic cancer epithelial cells.

Hsp90 is associated with IKK proteins, and PMA induces the dissociation of complex between Hsp90 and IKK-γ

The next important question was how sustained activation of PKC with PMA induced the IKK-β and IKK-γ protein degradation in colonic cancer epithelial cells. A recent study...
demonstrated that Hsp90 and Cdc37 are directly associated with kinase domain of IKK-α and IKK-β, and that they contribute to the stabilization, activation and/or translocation of these kinases (16,17). These results prompt us to test the possibility that Hsp90 may play a role in regulating PMA-induced IKK degradation in these cells. First, we examined whether Hsp90 and other members of Hsp families could associate with the endogenous IKK proteins in HCT-116 cells. As consistent with previous reports (16,17), IKK-α and IKK-β interacted with Hsp90, but not with other family members including Hsp27 and Hsp70 under non-stimulated conditions (Figure 4A) in HCT-116 cells. Furthermore, we found the strong interaction between IKK-γ and Hsp90 in the same conditions. Immunoprecipitation of these extracts with normal IgG followed by western blot analysis with IKK antibodies revealed no detectable interaction of these proteins, indicating the specificity of an association of IKK complex with Hsp90. To strengthen our findings, the interaction of IKK-γ and Hsp90 in colon cancer tissues was strong and that Hsp90 helps in maintaining the IKK complex stability and degradation after PMA treatment, suggesting that PMA treatment results specifically in decreased association of IKK-γ with Hsp90 in HCT-116 cells.

Enhanced expression of Hsp90 in colon cancer
Since Hsp90 helps in maintaining protein stability of several growth-promoting kinases, including IKK, v-Src and Raf, by forming a stable complex (17,37,38), the level of Hsp90 expression may have a close relationship with tumorigenesis of colon tissues. Therefore, the expression levels of Hsp proteins were compared by western blotting in normal and cancerous tissues from eight patients who underwent surgery for malignant colon cancer. As shown in Figure 5A, colon cancer tissues expressed Hsp90 at a dramatically higher level than the normal tissues for all samples. More importantly, the similar amount of expression of Hsp70 and Hsp27 between normal and cancerous tissues was detected, suggesting that Hsp90 expression become a promising marker for malignant colon cancer.

Inhibition of TNF-induced NF-κB activation after PMA treatment
Because colonic epithelial cells were shown to have an atypical IKK-β and IKK-γ degradation after PMA treatment

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### Table 1: Western Blotting Results

<table>
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<tr>
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<th>HCT-116</th>
<th>SW480</th>
<th>HM7</th>
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<tr>
<td><strong>PMA</strong></td>
<td>−</td>
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**Panel A:**
- **HCT-116**
  - **PMA:** −
  - **PMA:** +
- **SW480**
  - **PMA:** −
  - **PMA:** +
- **HM7**
  - **PMA:** −
  - **PMA:** +
- **LS 174T**
  - **PMA:** −
  - **PMA:** +

**Panel B:**
- **Hep 3B**
  - **PMA:** −
  - **PMA:** +
- **SNU-1**
  - **PMA:** −
  - **PMA:** +
- **SW 1990**
  - **PMA:** −
  - **PMA:** +
- **Capan-2**
  - **PMA:** −
  - **PMA:** +

**Observations:**
- IKK-γ and actin blots were used.
- Western blottings with anti-IKK-γ and anti-β-actin antibodies.
Inhibited TNF-induced IKK activation; even PMA alone slightly induced IKK activation. To determine whether PMA had any effect on other TNF-induced responses, we also examined TNF-induced JNK activation after PMA treatment. In contrast to IKK activation, TNF-induced JNK activation was unaffected by PMA (bottom panel of Figure 6A). Because PMA-induced IKK protein degradation was mediated by proteasome (Figure 2A), we asked whether prevention of IKK degradation by MG-132 was sufficient to recover TNF-induced NF-κB activation in the presence of PMA. As shown in Figure 6B, whereas PMA abolished TNF-induced IKK activation, MG-132 fully reverses PMA-mediated inhibition of IKK activation in response to TNF. As a control, the presence of MG-132 alone had minimal effect on IKK activation. To further confirm whether the inhibitory effect of PMA on the NF-κB signaling pathway is specific in colonic epithelial cells, we compared the effect of PMA on TNF-induced NF-κB activation in HCT-116 cells and Hep3B cells transiently transfected with a NF-κB-response reporter plasmid. Consistent with the results in Figure 6A, pretreatment of cells with PMA dramatically suppressed TNF-induced reporter activity in HCT-116 cells but not in Hep3B cells (Figure 6C). Since NF-κB activation protects cells against TNF-induced apoptosis and promotes cell survival and transformation, it may play a critical role in tumorigenesis. To determine the functional consequences of downregulation of NF-κB induced by PMA, we examined whether PMA can sensitize cells to TNF-induced apoptosis. HCT-116 cells were pretreated with PMA followed by TNF treatment. As shown in Figure 6D, although TNF or PMA alone only killed 7 and 15% of cells, respectively, the combination of PMA and TNF caused nearly 53% of cells to undergo apoptosis. As a control, a similar portion of cells died when pretreated with specific NF-κB inhibitor Bay-11. These data suggest that PMA can potentiate TNF-induced cell death in colonic epithelial cells through the downregulation of NF-κB signaling pathway.

Discussion

A number of previous studies have demonstrated that sustained activation of PKC with long-term treatment of

Fig. 4. Hsp90 is associated with IKK complex, and PMA causes a dissociation of the complex between Hsp90 and IKK-γ in HCT-116 cells. (A) HCT-116 cell extracts were immunoprecipitated with antibodies directed against Hsp27, Hsp70, Hsp90 or normal IgG. Immunoprecipitates were analyzed by western blotting with anti-IKK-α, anti-IKK-β and anti-IKK-γ antibodies (upper three panels). The efficiency of immunoprecipitants was measured with anti-Hsp27, anti-Hsp70 and anti-Hsp90 antibodies (bottom three panels). One percent of cell extracts from each sample was used as a control of protein input. (B) HEK293 cells were transfected with HA-tagged IKK-α, IKK-β and IKK-γ expression plasmids and flag-tagged Hsp-90 expression plasmid. Immunoprecipitates containing Hsp90 and IKK-γ were prepared with anti-flag and anti-HA antibodies, and immunoprecipitants were analyzed by western blotting with anti-HA and anti-flag antibodies. The efficiency of immunoprecipitants was measured with anti-flag and anti-HA antibodies (each bottom panel), and one percent of cell extracts from each sample was used as a control of protein input. IKK-γ association with Hsp90 is altered by PMA (C), but not by TNF (D). HCT-116 cells were treated with PMA (10 nM) or TNF (15 ng/ml) for various times as indicated, and cell extracts from each sample were immunoprecipitated with anti-IKK-γ antibody. Immunoprecipitants were analyzed by western blotting with anti-Hsp90, anti-Hsp70, anti-IKK-α, anti-IKK-β and anti-IKK-γ antibodies. One percent of cell extracts from each sample was used as a control of protein input.
PMA induces alterations of growth and differentiation in intestinal epithelial cells (26–28). The mechanism of this effect, however, has remained incompletely understood. Moreover, it has been unclear whether NF-κB signaling pathway might have a role in the epithelial cell differentiation stimulated by PMA. In the present study, we have demonstrated that long-term treatment of PMA downregulates the NF-κB signaling pathway through the degradation of IKK complex in several colonic epithelial cell lines, which distinguish these cells from other gastrointestinal tract lineages. We also found that a high level of expression of Hsp90 in colon cancer tissues, and an association of IKK-γ and Hsp90, was interfered by PMA in HCT-116 cells, resulting in IKK-γ and IKK-β destabilization and its subsequent proteasome-mediated degradation. As a consequence, long-term treatment of PMA selectively blocked TNF-induced NF-κB activation, therefore sensitizing cells to TNF-induced apoptosis.

Several lines of evidence have implicated aberrant alterations of PKC in the dysregulation of cellular functions, including regulation of cell balance and maturation that characterize colon cancer. For example, changes in the expression, biochemical activity, and/or subcellular distribution of specific isoforms of PKC have been associated with colonic malignant transformation in both human and experimental models of colon cancer (32,33,39). These findings suggest that PKC families are not only involved in the maintenance of normal colonic homeostasis but also contribute to the process of colonic carcinogenesis. In this regard, elucidation of signaling mechanism during epithelial differentiation with sustained activation of PKC will be critical to understand their roles in colonic tumorigenesis. In this study, we found that several colonic cancer cell lines showed an

Fig. 5. Overexpression of Hsp90 in colon cancer tissues. (A) Normal and cancer tissue extracts from eight patients who underwent surgery for malignant colon cancer were prepared as described in Materials and methods, and the expression levels of Hsp27, Hsp70 and Hsp90 were analyzed by western blotting with antibodies directed against each of the Hsp families and β-actin. (B) (A, B) IHC of Hsp90 in normal and carcinous tissues. Hsp90 was highly expressed in colon cancer cells (Ca) compared with faintly stained normal colonic mucosa (N). The expression of Hsp90 was observed mainly in the cytoplasm. (C,D) Negative isotype control with preimmune mouse IgG1. N, normal tissue of colon; Ca, cancer tissue of colon. Original magnification, A and C, 40×; B and D, 100×. (C) Enhanced association of IKK-α with Hsp90 in colon cancer tissues. The extracts from normal and malignant colon cancer tissues were immunoprecipitated with anti-Hsp90 antibody as described in the legend of Figure 4A, and immunoprecipitants were analyzed by western blotting with anti-IKK-α antibody.
altered regulation of NF-κB activation compared with the cells from other gastrointestinal tract. One of the interesting findings from this study is that long-term treatment of PMA can downregulate the NF-κB activation through the selective degradation of IKK-γ and subsequently IKK-β, indicating that suppressed NF-κB signaling pathway by sustained activation of PKC might have a role in the epithelial cell differentiation. However, it is unclear why sustained activation of PKC selectively causes the downregulation of NF-κB signaling pathway in colon epithelial cells. It has been reported that several colon cancer cell lines are unique in that their regulation of NF-κB response contradicts with the evidence obtained by studies using other cell types such as hematopoietic and mesenchymal cell lineages (40,41). Furthermore, recent study (42) also has made unexpected observation that proteasome inhibitors MG-132 or lactacystin induce IKK activation and NF-κB-dependent transcriptional activity in colon epithelial cell lines, HT-29 cells, as consistent with our findings. These results raised the possibility that some fundamental difference of NF-κB signaling pathway exists between colonic epithelial cells and other cell types.

Hsp90 is now known to play an important role in signal transduction networks. The chaperone, in concert with a host of co-chaperone protein partners, stabilizes its client proteins while keeping them in a conformation that is able to respond to appropriate stimuli. Several growth-promoting kinases, including v-Src, Raf and RIP, form a stable complex with Hsp90 (18,19,37,38). Furthermore, it has recently been reported that Hsp90 most probably serves a critical function both in facilitating biosynthesis of components of the IKK complex and in maintaining the mature forms of the kinase complex in a conformation that

Fig. 6. Inhibition of TNF-induced NF-κB activation after PMA treatment in HCT-116 cells. (A) PMA blocks TNF-induced IKK, but not JNK activation. HCT-116 cells were treated with TNF-α (15 ng/ml) for 10 min, PMA (10 nM) for 5 h or TNF (15 ng/ml) for 10 min after 5 h PMA (10 nM) pretreatment. Non-treated cells were used as a control. Cells were lysed and subjected to immunoprecipitation (IP) with anti-IKK-α or anti-JNK1 antibody, respectively. Immune complex kinase assay was performed with [γ-32P]ATP and GST-κB-(1-54) or GST-c-Jun (1-79) as an exogenous substrate. GST-κB-α and GST-c-Jun phosphorylation were assessed by SDS-PAGE and autoradiography (upper panel). IKK-α and JNK1 content were analyzed by western blotting with anti-IKK-α and anti-JNK1 antibody (lower panel). (B) Pretreatment of proteasome inhibitor MG-132 recovers PMA-mediated inhibition of IKK activation in response to TNF. HCT-116 cells were pretreated with MG-132 (5 μM) for 30 min and treated with TNF-α (15 ng/ml) for 10 min, PMA (10 nM) for 5 h, or TNF (15 ng/ml) for 10 min after 5 h PMA (10 nM). IKK activity and expression level of IKK-α were measured as described in A. (C) PMA blocks TNF-induced NF-κB transcriptional activation in colonic epithelial cells, but not in hepatocellular carcinoma cells. HCT-116 cells and Hep3B cells were transfected with the p2x NF-κB reporter and pRSV-β-gal plasmid. Twenty-four hours after transfection, cells were treated with or without PMA (10 nM) for 30 min, and then TNF-α (15 ng/ml) for 12 h. Luciferase assays were performed as described in the legend of Figure 1E, and the results for NF-κB activity are the average of three independent experiments. (D) PMA enhances the apoptotic effects of TNF. HCT-116 cells were pretreated with CHX (10 μg/ml), PMA (10 nM) or Bay-11 (5 μM) for 30 min, and then treated with TNF-α (15 ng/ml) for 12 h. Cells were trypsinized and collected in PBS buffer. The viability of cells was determined by trypan blue exclusion assay. Each bar represents the average of three independent experiments. Data are normalized to the rate of spontaneous cell death occurring in untreated cells.
allows for its eventual biochemical function and stability (17). These results led us to examine the role of Hsp90 on this PMA-induced IKK degradation. Although the Hsp90/ Cdc37 complex was previously demonstrated to bind to both IKK-α and IKK-β (16), we observed that Hsp90 strongly interacts with IKK-γ as well as IKK-α and IKK-β in colonic epithelial cells. More importantly, we found that high level of expression of Hsp90 and enhanced association with IKK in colon cancer tissues, and interaction between Hsp90 and IKK-γ, is regulated by PMA treatment, suggesting that Hsp90 is specifically involved in the regulation of PMA-induced NF-κB signaling pathway in these cells. Other Hsp families, Hsp27 and Hsp70, did not interact with IKK complex, indicating that not all members of Hsp9s are involved in this process.

Previous studies reported that Lys63(K63) or Lys(K6)-linked ubiquitination of IKK-γ may be involved in IKK activation through a proteasome-independent mechanism (36,43). However, given our findings that the pretreatment of proteasome inhibitor MG-132 antagonized PMA-induced IKK degradation and significantly recovered PMA inhibition of TNF-induced IKK activation in HCT-116 cells, it could be possible to induce the formation of polyubiquitin chain to IKK-γ or IKK-β after PMA treatment, leading to their subsequent recognition and degradation by the proteasome. Consistent with this idea, we found that IKK-γ was Lys48(K48)-linked polyubiquitinated during PMA treatment, and the ubiquitinated IKK-γ targeted for degradation to the proteasome. However, it is still unclear how PMA, unlike TNF, induced IKK-γ polyubiquitination, leading to degradation to proteasome. Since our observation that PMA induces the dissociation of complex between Hsp90 and IKK complex, and several other Hsp90 client proteins including IKK-β have been shown to be degraded by the ubiquitin–proteasome machinery (17), one possibility is that, in case of disruption of binding complex of IKKs from Hsp90 by PMA treatment, IKKs will be targeted to the proteasome through a unique K48-linked polyubiquitination chain in colonic epithelial cells. However, PKCs are not E3 ligase to ubiquitinate IKKs; identification of responsible proteins that might be able to function as an E3 to ubiquitinate IKK-γ/IKK-β in response to PKC activation will be critical for understanding the signaling mechanism of ubiquitination pathway. Therefore, further studies will be required to better understand its role for maintaining a stability of IKK complex following PKC activation.

We have shown that sustained activation of PKC with PMA downregulated NF-κB signaling pathway, and sensitized TNF-induced apoptosis in case of colon cancer epithelial cells by disrupting the interaction of IKK-γ with Hsp90. Of interest, several reports suggest that ulcerative colitis as a chronic inflammatory bowel disease is associated with an elevated risk for colon cancer (44,45) and IKK/ NF-κB pathway has an essential role during inflammation-associated tumor development (46). In addition, since our demonstration that differentiation of colonic epithelial cells by PKC shows an altered NF-κB regulation pattern compared with other hematopoietic and gastrointestinal cells, the understanding of NF-κB signaling pathway during differentiation of colon epithelial cells will be of great value for novel therapeutic approach from inflammatory bowel disease to colon cancer.

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References


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