

Regulation of phorbol ester-mediated TRAF1 induction in human colon cancer cells through a PKC/RAF/ERK/NF- κ B-dependent pathway

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Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are cytoplasmic adapter proteins that link a wide variety of cell surface receptors to the apoptotic signaling cascade. The purpose of this study was to delineate the signaling pathways and TRAF1 promoter elements responsible for phorbol ester-mediated TRAF1 induction in human colon cancers. Here, we found that the PKC activators, phorbol 12-myristate 13-acetate (PMA) and bryostatin I, induced TRAF1 mRNA expression; pretreatment with actinomycin D blocked PMA-mediated TRAF1 expression suggesting induction at the transcriptional level. In contrast, expression of other TRAFs (TRAF2, 3 and 4) was minimally altered by PMA. Various PKC isoform-selective inhibitors blocked PMA-mediated TRAF1 mRNA and promoter stimulation; rottlerin, a selective PKC δ inhibitor, had no effect suggesting that Ca²⁺-dependent PKC isoforms (e.g., PKC α and β I) play a role in TRAF1 regulation. In addition, the MEK/ERK inhibitors, PD98059 and UO126, suppressed PMA-stimulated TRAF1 promoter activity indicating a role for ERK in TRAF1 induction. Moreover, cotransfection of a dominant-negative Raf-1 (Raf-C4) significantly reduced PMA-stimulated TRAF1 promoter activity whereas transfection of dominant-negative Ras or treatment with Ras inhibitors had minimal to no effect on TRAF1 induction suggesting dependence on Raf, but not Ras, activation. Finally, site-specific mutagenesis of functional NF- κ B sites (particularly the most proximal site) in the TRAF1 promoter significantly decreased PMA-mediated promoter activity. In conclusion, our results demonstrate selective induction of TRAF1 in human colon cancer cells through a Ca²⁺-dependent PKC/Raf-1/ERK/NF- κ B-dependent pathway. *Oncogene* (2004) 23, 1885–1895 doi:10.1038/sj.onc.1207312 Published online 23 February 2004

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Introduction

Tumor necrosis factor (TNF) receptor associated factor (TRAF) family of proteins comprise a group of structurally related adaptor proteins that share a conserved carboxyl terminus, known as the TRAF domain, which mediates homo- and hetero-oligomerization, receptor binding and the association with a number of cytoplasmic proteins important for regulating cell survival and apoptosis (Arch *et al.*, 1998; Park *et al.*, 1999; Inoue *et al.*, 2000; Ni *et al.*, 2000; Tsao *et al.*, 2000; Wajant *et al.*, 2001). To date, six TRAF family proteins (TRAF1-6) have been described in mammals and characterized to variable degrees (Arch *et al.*, 1998; Inoue *et al.*, 2000; Wajant *et al.*, 2001). Specifically, the TRAF proteins serve to link the cytosolic domains of members of the TNF receptor (TNFR) superfamily and those of the Toll/IL-1R family to downstream protein kinases (e.g., JNK), ubiquitin ligases and other adaptor proteins (Deng *et al.*, 2000; Takaesu *et al.*, 2000; Bradley and Pober, 2001). Collective studies indicate that TRAF-mediated signals may directly induce cell survival, or, alternatively, TRAF interaction with intracellular proteins that trigger apoptosis may interfere with these proteins to induce programmed cell death (Arch *et al.*, 1998; Inoue *et al.*, 2000; Wajant *et al.*, 2001). Furthermore, TRAF proteins may be capable of fine tuning cellular responses to signals regulating cellular survival and death in a highly cell-dependent manner. The expression patterns of the TRAF proteins are strikingly different, further supporting independent and cell type-specific regulation of each TRAF protein (Rothe *et al.*, 1994; Mosialos *et al.*, 1995; Regnier *et al.*, 1995; Song and Donner, 1995; Cao *et al.*, 1996; Ishida *et al.*, 1996).

The *in vivo* expression of TRAF1 is the most restricted among the TRAFs; TRAF1 mRNA levels are found at readily detectable levels only in the spleen, lung and testis, unlike other TRAF proteins which are more ubiquitously expressed (Rothe *et al.*, 1994; Mosialos *et al.*, 1995; Zapata *et al.*, 2000). Furthermore, a marked induction of TRAF1 mRNA and protein levels occur in T and B cells by a variety of stimuli that induce the transcription factor NF- κ B (Schwenzer *et al.*, 1999; Wajant *et al.*, 2001). In this regard, analysis of the promoter region of the TRAF1 gene has demonstrated

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the presence of multiple NF- κ B-binding sites (Schwenzer *et al.*, 1999). TRAF1 levels are also typically increased in B-cell malignancies including non-Hodgkin's lymphomas and chronic lymphocytic leukemias as well as the Reed–Sternberg cells of Hodgkin's disease (Irmeler *et al.*, 2000; Wajant *et al.*, 2001). TRAF1 can be recruited to the TNFR1 and TNFR2 through interaction with TNFR-associated death domain protein (TRADD) and TRAF2, respectively, where it is also found associated with c-IAP1 (cellular inhibitor of apoptosis protein 1) and c-IAP2, which can suppress TNF- α -dependent caspase-8 activation, thereby preventing apoptosis (Arch *et al.*, 1998; Inoue *et al.*, 2000; Wajant *et al.*, 2001). In addition, TRAF1 might sequester TRADD, RIP or other proapoptotic proteins that can act to further enhance cell survival.

Although the regulation of TRAF expression has been relatively well characterized in lymphoid cells and lymphoid malignancies, the regulation of expression in solid cancers is not well defined. Our laboratory is interested in mechanisms regulating the expression of the apoptotic-related genes in colorectal cancers (Hernandez *et al.*, 2001; Wang *et al.*, 2002). Therefore, the purpose of this present study was to analyse the mechanisms regulating TRAF gene expression in colorectal cancers. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), were originally described as tumor promoters but these agents can also modulate a number of different cellular processes such as growth, differentiation, apoptosis and gene transcription through the protein kinase C (PKC) signaling pathway. PMA, which can substitute for diacylglycerol (the endogenous PKC activator), has been utilized as a model agent to analyse the potential mechanisms used by growth factors and hormones to modulate cell growth and differentiation in a number of cell types (Nishizuka, 1995; Jaken, 1996; Parekh *et al.*, 2000; Lee *et al.*, 2002). Here, we found that PMA induced TRAF1 expression in a dose- and time-dependent fashion. Various PKC isoform-selective inhibitors blocked PMA-mediated TRAF1 mRNA and gene promoter stimulation; rottlerin, a selective PKC δ inhibitor, had no effect suggesting that Ca²⁺-dependent PKC isoforms (e.g., PKC α and β I) play a role in TRAF1 regulation. In addition, inhibition of MEK/ERK suppressed PMA-stimulated TRAF1 promoter activity indicating an important role for ERK in TRAF1 induction. Moreover, cotransfection of dominant-negative Raf-1 significantly reduced PMA-stimulated TRAF1 promoter activity whereas transfection of dominant-negative Ras or treatment with Ras inhibitors had minimal to no effect on TRAF1 induction suggesting dependence on Raf, but not Ras, activation. Finally, site-specific mutagenesis of functional NF- κ B sites in the TRAF1 promoter significantly decreased PMA-mediated promoter activity. Taken together, our results demonstrate selective induction of TRAF1 in human colon cancer cells through a Ca²⁺-dependent PKC/Raf-1/ERK/NF- κ B-dependent pathway.

Results

Phorbol ester, PMA, induces TRAF1 expression in human colon cancers

The TRAF proteins are critical for regulating survival, proliferation and stress responses in certain cell types (Arch *et al.*, 1998; Takaesu *et al.*, 2000; Bradley and Pober, 2001); however, the mechanisms regulating TRAF expression in solid cancers (e.g., colorectal cancers) is not well known. Initially, we determined the effects of the phorbol ester, PMA, on TRAF mRNA expression in the SW480 human colon cancer cell line by RNase protection assays using a commercially available multi-template set (hAPO-5; Pharmingen) containing specific probes for TRAF (1, 2, 3, 4), c-IAP1, c-IAP2, XIAP and testosterone-repressed message-2 (TRPM-2); L32 and GAPDH are included to control for RNA loading. PMA stimulates TRAF1 expression in a dose- and time-dependent fashion (Figure 1a). PMA stimulates TRAF1 expression starting at a dosage of 4 nM (lane 3, Figure 1a). Increased expression was noted at 2 h after PMA treatment with peak expression at 4 h; expression of TRAF1 returned to control levels at 24 h (lanes 9–14, Figure 1a). In contrast, expression levels of TRAF2, TRAF3 and TRAF4 were minimally affected, if at all. In addition, the expression of TRPM2 and

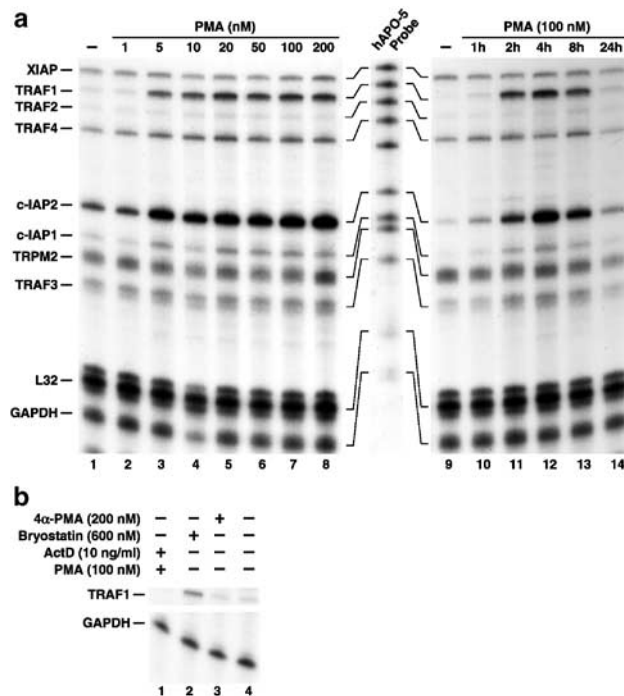


Figure 1 Induction of TRAF1 mRNA expression by PMA. (a) SW480 human colon cancer cells were treated with various concentrations of PMA (lanes 1–8) or with PMA (100 nM) over a time course (lanes 9–14). RNA was isolated and analysed by RNase protection using a labeled multi-template set (hAPO-5; Pharmingen). (b) SW480 cells were pretreated with actinomycin D (ActD) prior to PMA, treated with bryostatins or 4 α -PMA, a negative control for PMA. RNA was extracted and analysed by RNase protection; GAPDH is included as a control for RNA loading

TRPM3 and XIAP was minimally altered. PMA also stimulates expression of c-IAP1 and c-IAP2, two members of the Inhibitors of Apoptosis (IAP) family; the mechanisms for this induction are currently being evaluated in our laboratory (Wang QD, preliminary results).

We next determined whether the induction of TRAF1 expression by PMA is through increased transcription using actinomycin D (ActD; 10 ng/ml), which inhibits DNA-primed RNA polymerase (Betzel *et al.*, 1993; Wu and Yung, 1994), as pretreatment (lane 1, Figure 1b). ActD blocked PMA-mediated TRAF1 mRNA induction suggesting transcriptional regulation. To further confirm the effects of PMA on TRAF1 expression, SW480 cells were treated with bryostatin (600 nM), a structurally unique marine natural product that binds and activates PKC at picomolar concentration (Henings *et al.*, 1987), and 4 α -PMA, a negative control for PMA. Similar to PMA, bryostatin induced TRAF1 expression (lane 2, Figure 1b). In contrast, 4 α -PMA had no effect (lane 3, Figure 1b), thus further confirming the effect of PMA and suggesting the role of PKC in the PMA-mediated TRAF1 induction in SW480 cells.

Induction of TRAF1 through a PKC-mediated effect in human colon cancer cells

To further identify the signaling pathways contributing to TRAF1 induction, SW480 cells were pretreated for 20 min with GF109203x (GFx; 2 μ M), a highly selective cell-permeable PKC inhibitor that acts as a competitive inhibitor for the ATP-binding site of PKC (Gekeler *et al.*, 1996; Ku *et al.*, 1997), and then treated with either PMA (100 nM) or vehicle (i.e., DMSO) for 4 h (Figure 2a). GFx completely blocked TRAF1 mRNA and protein induction by PMA as demonstrated by RNase protection and Western blot, respectively. In contrast, treatment with the PKA inhibitor, H89, had no effect on TRAF1 induction (data not shown). As a further indication of the role of PKC, SW480 cells were treated with PMA (1 μ M) for 24 h to exhaust PMA-sensitive PKCs (Fang *et al.*, 2002), washed with PBS and then treated with PMA (100 nM) for 4 h (Figure 2b). Following chronic PMA treatment, TRAF1 expression was blocked in response to treatment with 100 nM PMA, further confirming that PMA-sensitive PKCs mediate the activation of TRAF1.

To determine whether the effects of PMA were specific to the SW480 cell line or, alternatively, whether TRAF1 is induced by PMA in other human colon cancer cells, we treated the colon cancer cell lines SW620, DLD-1, LoVo, and HCT116, HT29, Caco-2, CoLo201 and CoLo205 with PMA (100 nM), either alone or after pretreatment with GFx, and analysed RNA by RNase protection (Figure 2c). TRAF1 induction was noted in the SW620, DLD-1, LoVo and HCT116 cancer cell lines; this induction was blocked by GFx. In contrast, induction of TRAF1 mRNA was not detected in Caco-2, CoLo201, CoLo205 or HT29 cells using RNase protection analysis (data not shown). These cell-specific differences noted in TRAF1 induc-

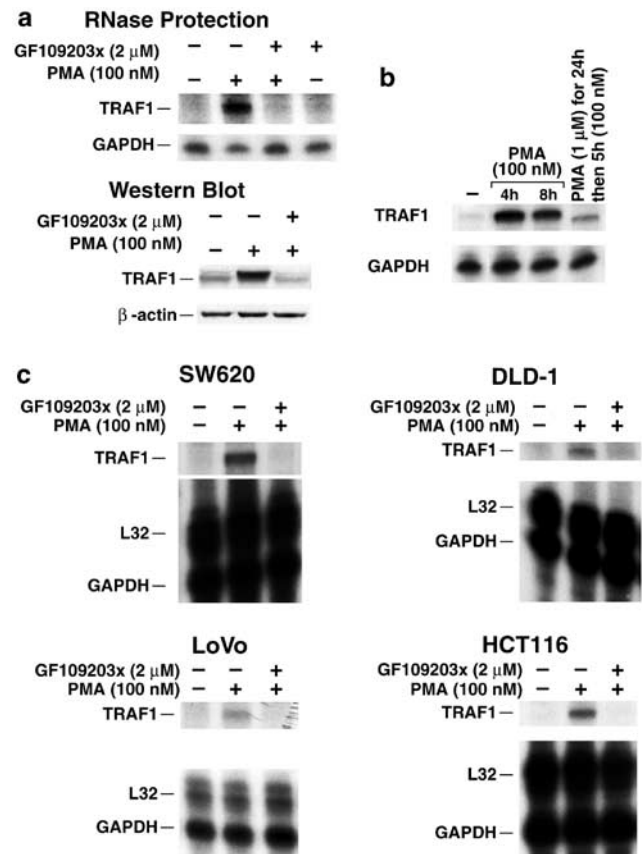


Figure 2 Inhibition of PKC blocks PMA-mediated TRAF1 induction. (a) SW480 cells were pretreated with the PKC inhibitor, GF109203x, and then treated with PMA; cells were extracted for RNA and protein to determine TRAF-1 expression by RNase protection and Western blot, respectively. (b) SW480 cells were treated with PMA (1 μ M) for 24 h to deplete PMA-sensitive PKCs and then treated with PMA (100 nM) for 5 h; RNA was extracted and analysed by RNase protection. TRAF1 expression was compared to induction by PMA alone. (c) To confirm TRAF1 induction in other human colon cancer cells, SW620, DLD-1, LoVo and HCT116 cells were treated with PMA (100 nM) with or without pretreatment with the PKC inhibitor, GF109203x (2 μ M). RNA was extracted and analysed by RNase protection

tion may relate to differences in the genetic profile of these tumors. Alternatively, although RNase protection provides a sensitive method to detect changes in gene expression, more sensitive methods may be required to detect TRAF1 mRNA changes in other colon cancer cells. For example, in preliminary studies, we have utilized RT-PCR and detected PMA-mediated TRAF1 mRNA induction in Caco-2 cells (data not shown).

Ca²⁺-dependent PKCs play a role in TRAF1 stimulation by PMA

To examine the PKC isoforms contributing to TRAF1 induction by PMA, SW480 cells were pretreated with varying concentrations of isoform-selective PKC inhibitors (Figure 3a). GFx (which inhibits PKC α , β I, β II, γ , δ and ζ), G δ 6983 (which inhibits isoforms α , β , γ , δ and ζ) and Ro-31-8220 (which inhibits isoforms α , β I,

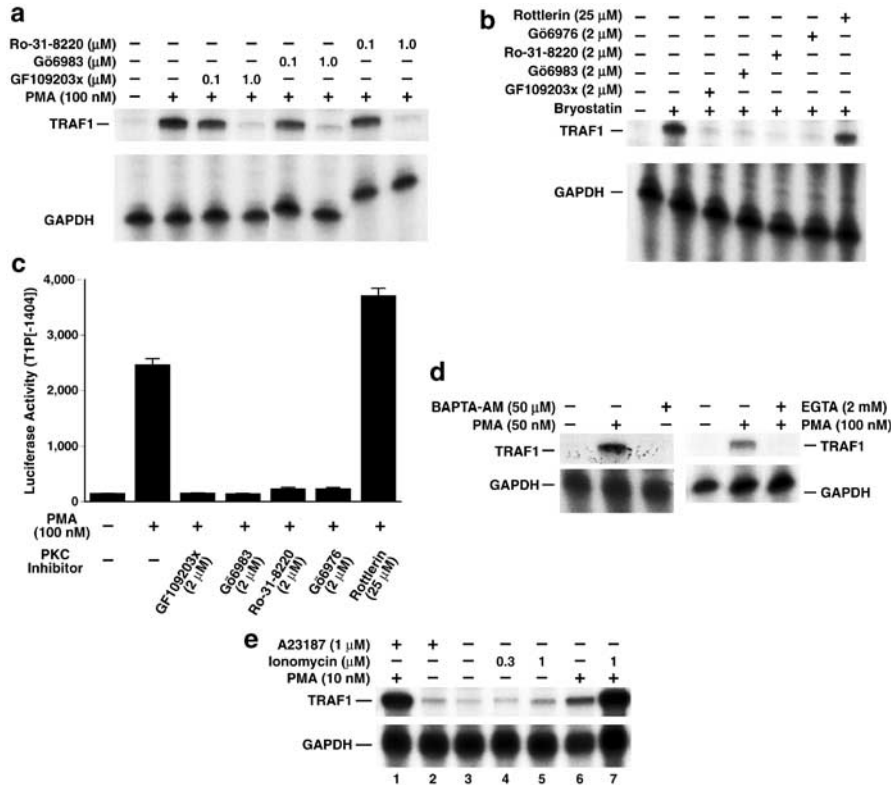


Figure 3 Ca^{2+} -dependent PKC isoforms contributing to TRAF1 induction. (a) SW480 cells were treated with PMA with or without various isoform-selective PKC inhibitors (0.1 and 1.0 μM). RNA was extracted and analysed by RNase protection. (b) SW480 cells were treated with bryostatin, with or without various isoform-selective PKC inhibitors. RNA was extracted and analysed by RNase protection. (c) SW480 cells were transiently transfected with a promoter construct containing 1404 bp of the TRAF1 proximal promoter linked to luciferase (T1P[-1404]) and a construct (pRL-TK) containing Renilla luciferase. Approximately, 40 h after transfection, cells were pretreated for 0.5 h with the PKC inhibitors and then treated with PMA (100 nM). Cells were harvested 8 h after PMA treatment; luciferase activity was assayed and transfection efficiency corrected using Renilla luciferase. (Representative experiment shown from three separate experiments utilizing 3 wells/construct.) (d) SW480 cells were pretreated with the permeable Ca^{2+} chelator, BAPTA-AM, or the nonpermeable Ca^{2+} chelator, EGTA, and then treated with PMA. RNA was extracted and analysed by RNase protection. (e) SW480 cells were pretreated with the calcium ionophores ionomycin (either 0.3 or 1 μM) or A23187 (1 μM) and then treated with PMA. RNA was extracted and analysed by RNase protection

βII , γ and μ), at a concentration of 1 μM , blocked the induction of TRAF1 by PMA. Inhibition of TRAF1 was also demonstrated using Gö6976 (inhibits PKC isoforms α , βI and μ) but not with rottlerin, which is a more selective PKC δ inhibitor (data not shown). Similar to the effects noted with PMA, the PKC inhibitors GFx, Gö6983, Ro-31-8220 and Gö6976, but not rottlerin, blocked TRAF1 mRNA induction by bryostatin (Figure 3b).

To further confirm that the effects of PMA on TRAF1 are through a transcriptional mechanism and determine the effects of these PKC inhibitors on TRAF1 promoter activity, SW480 cells were transiently transfected with a promoter construct containing 1404 bp of the TRAF1 proximal promoter linked to luciferase (T1P[-1404]). Treatment with PMA dramatically induced luciferase activity, which was blocked by all of the PKC inhibitors except for the PKC δ -selective inhibitor, rottlerin (Figure 3c). Taken together, these findings corroborate the results of TRAF1 mRNA induction and further suggest that the effects are mediated through an increase in transcription.

The dependence of Ca^{2+} for TRAF1 stimulation by PMA was examined by incubating SW480 cells with BAPTA-AM (50 μM), a cell-permeable Ca^{2+} chelator (Billman, 1993; Jiang *et al.*, 1994; Mulvaney and Roberson, 2000), or EGTA (2 mM), a nonpermeable Ca^{2+} chelator (Mulvaney and Roberson, 2000), to chelate intracellular and extracellular Ca^{2+} , respectively (Figure 3d). Either BAPTA-AM or EGTA completely inhibited the induction of TRAF1 mRNA by PMA demonstrating the importance of Ca^{2+} in the induction of TRAF1. Furthermore, treatment with calcium ionophores, ionomycin or A23187, which increase intracellular Ca^{2+} (Lee, 1993), resulted in a significantly enhanced induction of TRAF1 in combination with PMA (10 nM) (Figure 3e; lanes 1 and 7) compared with PMA treatment alone (lane 6). Ionomycin or A23187 alone (lanes 2 and 5) resulted in a slight increase in TRAF1 expression compared with vehicle treatment (lane 3). Collectively, these results suggest that Ca^{2+} -dependent PKC isoforms (most likely PKC α and/or βI) play a critical role in the regulation of TRAF1 by PMA. In addition, there is also the possibility that TRAF1

induction in colon cancer cells occurs, in part, through activation of Ca^{2+} /calmodulin-dependent protein kinases.

Involvement of MEK/ERK in TRAF1 stimulation by PMA

The effect of PKC may be mediated through the MAPK or PI3K signaling pathways (Hipp *et al.*, 2002); therefore, we next determined the potential role of these pathways on PMA-mediated TRAF1 induction (Figure 4). To first determine the role of ERK1/2, acting downstream of PKC, cells were pretreated with the MEK inhibitors PD98059 or UO126 and then treated with PMA (100 nM) for 4 h. Both inhibitors significantly attenuated, but did not completely block, TRAF1 mRNA induction by PMA (Figure 4a). As a further confirmation of the effect of PMA on ERK stimulation, Western blots using antibody specific for phosphorylated ERK1/2 showed a marked early (by 5 min) induction of ERK1/2 phosphorylation which was blocked by either the PKC inhibitor GFx or PD98059 (data not shown).

In contrast, pretreatment with the p38/MAPK inhibitor, SB203580, or the PI3K inhibitor, wortmannin,

or the JNK inhibitor SP600125 had no effect on the induction of TRAF1 (data not shown). To further corroborate findings at the level of promoter activity, we again transfected the TRAF1-luciferase vector into SW480 cells; the cells were treated with PMA or vehicle with or without the MEK inhibitors PD98059 or UO126 (Figure 4b). PMA alone stimulates TRAF1 luciferase activity, which was significantly suppressed by either of the MEK inhibitors. Similar to findings in Figure 4a, the MEK inhibitor did not block induction of luciferase activity entirely, suggesting that MEK-dependent and -independent pathways mediate TRAF1 induction by PMA.

Raf-1, but not Ras, mediates TRAF1 activation by PMA

PMA/PKC can activate MEK/ERK through Ras-dependent or -independent pathways (Ueda *et al.*, 1996; Verin *et al.*, 2000; Vuong *et al.*, 2000; Zhao *et al.*, 2001). We next determined the role of Ras and Raf in TRAF1 stimulation. SW480 cells were transfected with either a dominant-negative Raf-1 (Raf-C4), dominant-negative Ras (Ras-N17), or empty vector, to control for DNA concentration, in combination with the TRAF1 promoter construct. After 24 h incubation, cells were treated with either PMA or vehicle (i.e., DMSO) and luciferase activity was measured. Cotransfection with Raf-C4 suppressed PMA-stimulated TRAF1 promoter activity compared with transfection with the empty vector (Raf-C4pm17) (Figure 5a). In contrast, coexpression of the dominant-negative Ras (Ras-N7) did not decrease PMA-mediated TRAF1 promoter induction (Figure 5b).

To further confirm the results regarding the role of Ras, the farnesyltransferase (FTase) inhibitor FPT-I3 was used. Prenylation includes farnesylation and/or geranylgeranylation (Adjei, 2001). Farnesylation of Ras is the most critical step in post-translational processing and is required for Ras function (Adjei, 2001). As shown in Figure 5c, FPT-I3 had no effect on TRAF1 stimulation by PMA (lane 5), however, the GGTase inhibitors, GGTI-2133 and GGTI-298, attenuated the induction of TRAF1 expression (lanes 3 and 4). In addition, another GGTase inhibitor, GGTI-286, demonstrated a similar attenuation of TRAF1 expression (data not shown).

Although inhibition of farnesylation with the FTase inhibitor had no effect on PMA-mediated TRAF1 stimulation, a number of studies have shown that K-Ras can be geranylgeranylated and, therefore, required for effective Ras signaling (Gutierrez *et al.*, 1989; Adjei, 2001). To determine whether K-Ras may play a role in TRAF1 induction, Caco-2 cells, stably transfected with activated K-Ras (Taylor *et al.*, 2000), were treated with PMA and analysed by RNase protection; there was no TRAF1 induction with PMA treatment (data not shown). As a corollary study, HCT116 and DLD-1 cells, K-Ras disrupted HCT116 and DLD-1 (Okumura *et al.*, 1999), were treated with PMA and, similar to wild-type HCT116 and DLD-1 cells (Figure 2c), TRAF1 induction was demonstrated (data not shown). Taken

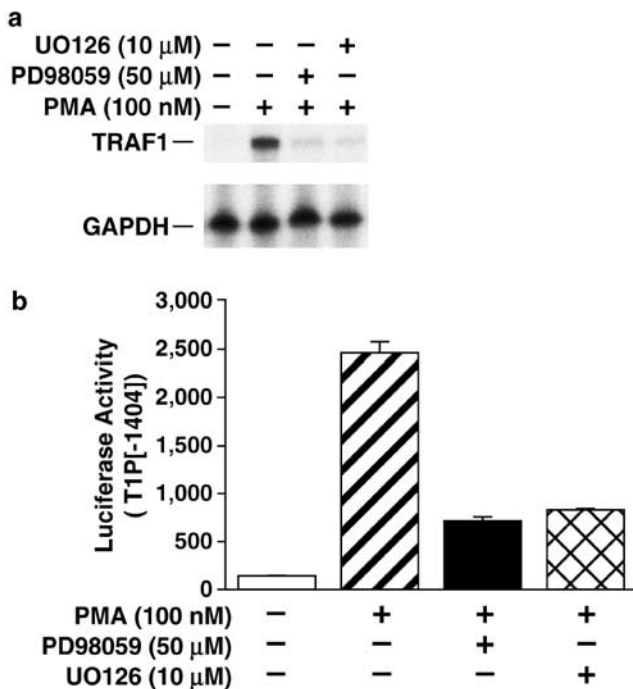


Figure 4 Induction of TRAF1 by PMA is dependent on ERK. (a) SW480 cells were pretreated with the MEK/ERK inhibitors, either PD98059 (50 μ M) or UO126 (10 μ M), and then treated with PMA (100 nM). RNA was extracted and analysed by RNase protection. (b) SW480 cells were transiently transfected with the TRAF1 promoter-luciferase construct [TIP(-1404)] along with the Renilla luciferase plasmid (pRL-TK). Approximately, 40 h after transfection, cells were pretreated with PD98059 or UO126 for 0.25 h followed by PMA (100 nM). Cells were harvested 6 h after PMA treatment and luciferase activities measured. (Representative experiment shown from three separate experiments utilizing 3 wells/construct.)

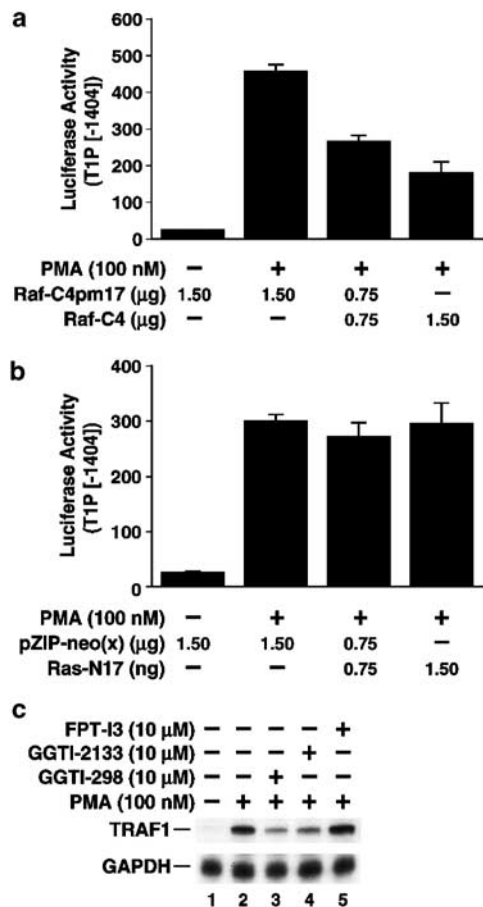


Figure 5 Induction of TRAF1 is dependent on Raf, but not Ras. (a) SW480 cells were cotransfected with the TRAF1 promoter-luciferase construct (T1P[-1404]) and the Raf-1 dominant-negative mutant (Raf-C4) or the control plasmid (Raf-C4pm17); transfection efficiency was controlled by transfection with the Renilla luciferase vector (pRL-TK). Cells were harvested 40 h after transfection and luciferase activities measured. (b) SW480 cells were cotransfected with T1P(-1404) and the Ras dominant-negative mutant (Ras-N17) or the empty vector pZIP-neo(x); transfection efficiency was controlled by transfection with the pRL-TK plasmid. Cells were harvested 40 h after transfection and luciferase activities measured. (c) SW480 cells were pretreated with the farnesyltransferase inhibitor, FPT-I3, which blocks farnesylation of Ras, or GGTase inhibitors, GGTI-2133 or GGTI-298 for 1 h and then treated with PMA for 4 h. RNA was extracted and analysed by RNase protection. (Representative experiment shown from three separate experiments utilizing 3 wells/construct.)

together, these findings further support the supposition that Ras is not required for TRAF1 induction in colon cancers. Moreover, our results, utilizing the GGTase inhibitors, indicate the possible involvement of other geranylgeranylated small G proteins, such as RhoA, Rap1 or Rac1 (Schaber *et al.*, 1990; Moores *et al.*, 1991; James *et al.*, 1995; Zhang and Casey, 1996; Finder *et al.*, 1997), in PMA-mediated TRAF1 induction.

NF-κB is required for TRAF1 induction by PMA

In some reports, the transcription factor, NF-κB, has been shown to be a downstream target gene of MEK/

ERK (Jiang *et al.*, 2001; Kurland *et al.*, 2003). NF-κB modulates TRAF1 activation by TNF ligands and other cytokines (Wang *et al.*, 1998a). To determine whether NF-κB plays a role in the PKC-mediated induction of TRAF1, we pretreated SW480 cells with MG132, a proteasome inhibitor (Hipp *et al.*, 2002), curcumin, an active yellow pigment of turmeric and curry which inhibits PMA-induced NF-κB activation (Schwenzer *et al.*, 1999), or gliotoxin, which inhibits NF-κB activation in B and T cells (Pahl *et al.*, 1996; Han *et al.*, 2002) (Figure 6a). Pretreatment with MG132 completely blocked PMA-mediated TRAF1 induction (lane 3, Figure 6a); both curcumin and gliotoxin markedly attenuated TRAF1 mRNA induction (lanes 4 and 5, Figure 6a).

The TRAF1 proximal promoter contains three functional NF-κB sites, κB1, κB3 and κB5 (Figure 6b) (Schwenzer *et al.*, 1999). Transient transfection analysis demonstrated induction of TRAF1 promoter activity using the wild-type construct (-1404) containing all of the NF-κB binding sites; deletion of the κB1 site (-424) markedly abrogated TRAF1 luciferase activity with PMA treatment and deletion of all of the NF-κB sites completely blocked PMA-mediated TRAF1 promoter induction (Figure 6c). To further define the role of the three NF-κB sites in TRAF1 expression, site-specific mutagenesis of these three sites was performed (Figure 6d). Mutagenesis of κB3 and κB1 sites attenuated TRAF1 promoter induction by PMA to variable degrees with almost complete inhibition of TRAF1 promoter induction noted with mutation of the κB1 site. Mutation of the κB5 site completely blocked PMA-mediated TRAF1 induction. These findings demonstrate the functional importance of NF-κB binding sites, particularly the κB1 and κB5 sites, in TRAF1 induction by PKC in human colon cancer cells.

NF-κB is regulated by IκB proteins which sequester NF-κB in the cytoplasm (Chen *et al.*, 1999; Karin and Ben-Neriah, 2000). To further demonstrate PMA-mediated TRAF1 induction is dependent on NF-κB, we next transfected SW480 cells with the wild-type (-1404) TRAF1 promoter construct in combination with the IκB super-repressor (IκB α M), a vector over-expressing IKK β (the major IKK activated in response to stimuli), IKK β -KM (a kinase inactive mutant of IKK β) or empty vector (Figure 6e). Induction of TRAF1 promoter activity was noted in SW480 cells cotransfected with the empty vector and treated with PMA. In contrast, cotransfection with the IκB super-repressor, IκB α M, decreased basal TRAF1 promoter activity by 75% compared with empty vector and completely blocked PMA-mediated TRAF1 induction. Cotransfection with IKK β stimulated TRAF1 promoter activity in the absence of PMA; treatment with PMA further augmented TRAF1 promoter induction by IKK β . Finally, cotransfection with IKK β -KM had a similar effect as the IκB super-repressor with complete inhibition of PMA-mediated TRAF1 induction. Together, these results, utilizing a variety of complementary techniques, demonstrate the importance of NF-κB in the induction of TRAF1 in human colon cancers.

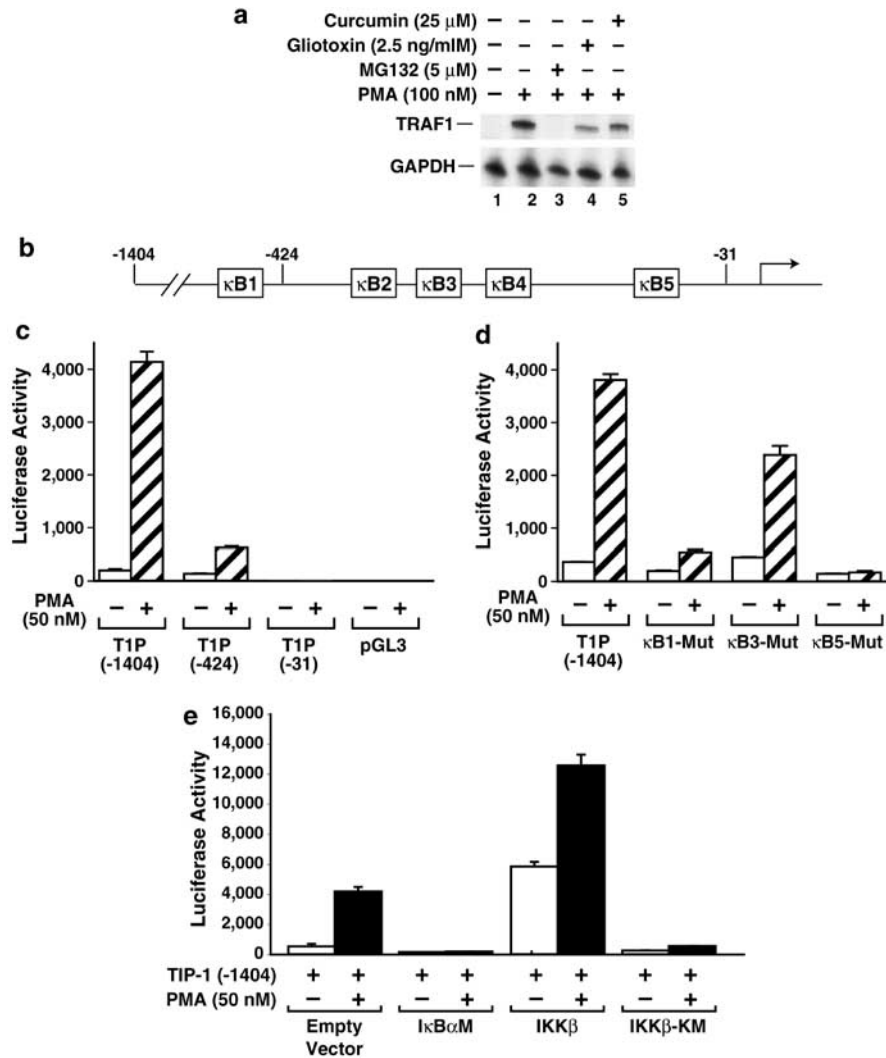


Figure 6 Analysis of NF- κ B sites critical for TRAF1 induction. (a) SW480 cells were pretreated with curcumin, gliotoxin or MG132 for 1 h and then treated with PMA (100 nM). Cells were harvested 4 h later, extracted for RNA and analysed by RNase protection. (b) Schematic diagram showing the location of five NF- κ B sites present in the proximal TRAF1 promoter (Schwenzer *et al.*, 1999). (c) SW480 cells were transiently transfected with 5' deletion constructs of the TRAF1 promoter linked to the luciferase reporter gene or the empty vector (pGL3). At 34 h after transfection, cells were treated with PMA (50 nM) or vehicle and harvested 8 h later. Luciferase activity was measured; transfection efficiency was corrected by cotransfection with a plasmid containing Renilla luciferase. (d) SW480 cells were transfected with the wild-type TRAF1 promoter construct (TIP[-1404]) or constructs containing site-specific mutations of either the κ B1, κ B3 or κ B5 sites. At 34 h after transfection, cells were treated with PMA (50 nM) or vehicle and harvested 8 h later. Luciferase activity was measured; transfection efficiency was corrected by cotransfection with a plasmid containing Renilla luciferase. (e) SW480 cells were cotransfected with the wild-type TRAF1 promoter construct (TIP [-1404]) and the I κ B super-repressor (I κ B α M), a vector overexpressing IKK β or the kinase-inactive mutant IKK β (IKK β -KM) and treated with PMA (50 nM) or vehicle; transfection efficiency was controlled by transfection with the pRL-TK plasmid. Cells were harvested 40 h after transfection and luciferase activities measured. (Representative experiment shown from three separate experiments utilizing 3 wells/construct.)

Discussion

TRAF1 is an antiapoptotic component of the intracellular signaling pathway of the TNFR family. Although it has been shown that TRAF1 can be induced by selected stimuli (e.g., IL-1 and members of the TNF ligand family), and activated T and B cells (Mosialos *et al.*, 1995; Lee *et al.*, 1996; Carpentier and Beyaert, 1999; Dunn *et al.*, 1999; Schwenzer *et al.*, 1999; Wajant *et al.*, 2000), the intracellular mechanisms regulating TRAF1 gene expression remain largely undefined.

Furthermore, the regulation of TRAF1 expression in human colon cancer cells has not been well investigated. In our present study, we found that activation of PKC by either PMA or bryostatin strongly stimulates TRAF1 promoter activity and TRAF1 gene and protein expression in colon cancer cell lines. This induction was specific for TRAF1 since other TRAF family members demonstrated minimal to no induction of expression after PMA or bryostatin I treatment. In addition, induction of TRAF1 expression was not noted in all colon cancer cell lines. The reason for the specificity of

TRAF1 induction in certain colon cancer cell lines is not known but may be related to differences in genetic profiles in the tumors or, possibly, the sensitivity of the assays.

To delineate the signaling pathways involved in PMA-mediated TRAF1 stimulation, the effects of various chemical PKC inhibitors were analysed. Isoform-selective PKC inhibitors GFx, Gö6983, Gö6976 and Ro-31-8220, all of which inhibit PKC α , β 1, blocked TRAF1 induction by PMA. In contrast, the PKA inhibitor, H89 and the PKC δ -selective inhibitor Rotlerin, had no effect on PMA induction of TRAF1. Consistent with our results, Carpentier *et al.* (Carpentier and Beyaert, 1999) found that PMA-induced TRAF1 protein expression was inhibited by Ro-31-8220 in HeLa cervical cancer cells. Collectively, these results demonstrate that the induction of TRAF1 by PMA is through the activation of conventional PKCs. Moreover, our results utilizing the calcium chelator, BAPTA-AM, and EGTA, or calcium ionophores A23187 and ionomycin, further demonstrate the role of Ca²⁺-dependent PKC isoforms (conventional PKCs) in TRAF1 induction by PMA.

PKC-mediated activation of the Ras/Raf/MEK/ERK signaling pathway has been well-characterized, but the exact nature and order of events leading to Raf activation remain to be fully elucidated. The ability of dominant-negative Ras mutants to inhibit PMA-mediated Raf activation has been reported to be cell line-dependent. PKC can activate Raf directly by phosphorylation or, alternatively, it has been proposed that PKC can activate Ras, which subsequently leads to Raf and ERK activation. For example, PMA/PKC α directly phosphorylates and activates Raf1 in NIH-3T3 fibroblasts (Kolch *et al.*, 1993). In addition, PKC, independent of Ras, plays a critical role in angiotensin-induced Raf1 and MEK activation in cardiac myocytes (Zou *et al.*, 1996), and PKC δ activates the MEK/ERK pathway, independent of Ras but dependent on Raf activation, in COS and NIH-3T3 cells (Ueda *et al.*, 1996; Seternes *et al.*, 1999). In contrast, other studies have shown that PMA/PKC regulates downstream gene induction and functions through sequential induction of Ras, Raf and MEK (Chiloeches *et al.*, 1999; Verin *et al.*, 2000; Vuong *et al.*, 2000); these effects appear to be dependent on cellular context. In our present study, cotransfection with a dominant-negative Raf effectively inhibited TRAF1 promoter induction by PMA. This inhibition occurred in a dose-dependent fashion, further demonstrating Raf participation in PMA-mediated TRAF1 stimulation. In contrast, cotransfection with a dominant-negative Ras construct had no effect on TRAF1 promoter induction suggesting that the effects of PMA on TRAF1 expression are independent of Ras. To further confirm the role of Ras in TRAF1 stimulation by PMA, the FTase inhibitor, FPT-I3, and GGTase inhibitors were utilized. FPT-I3 inhibits Ras farnesylation, the most critical step in post-translational processing, and is required for Ras function (Gutierrez *et al.*, 1989; Wang *et al.*, 1998b; Adjei, 2001). Consistent with the absence of TRAF1 inhibition by the dominant-

negative Ras construct, FPT-I3 had no effect on TRAF1 gene induction by PMA.

Interestingly, all of the GGTase inhibitors significantly inhibited PMA-mediated TRAF1 induction. Both GGTI-298 and GGTI-2133 attenuated PMA-induced TRAF1 expression whereas GGTI-286 effectively blocked TRAF1 induction. Since it has been shown that K-Ras can be geranylgeranylated and is crucial for effective Ras signaling (James *et al.*, 1995, 1996; Lerner *et al.*, 1995; Zhang and Casey, 1996; Lerner *et al.*, 1997), we further analysed the potential role of K-Ras, which is the most frequently mutated form of Ras in human cancers (Adjei, 2001), for PMA-stimulated TRAF1 expression in colon cancers. Both parental Caco-2 cells and Caco-2 cells stably transfected with activated K-Ras were treated with PMA; TRAF1 induction was not detected in either cell line. Furthermore, K-Ras-disrupted HCT116 and DLD-1 cells were treated with PMA and, similar to parental cells, TRAF1 induction was noted in both cell lines. Together, our results indicate that PMA/PKC-mediated TRAF1 stimulation is Raf-dependent but Ras-independent. We speculate that other geranylgeranylated small G proteins, such as RhoA, Rap1 or Rac1 (Schaber *et al.*, 1990; Moores *et al.*, 1991; James *et al.*, 1995; Zhang and Casey, 1996; Finder *et al.*, 1997), may be involved in PMA-mediated regulation of TRAF1.

MEK1/2 is an important downstream effector of c-Raf (Dhillon *et al.*, 2003). Therefore, to explore the possibility that the c-Raf/MEK/MAPK pathway contributes to PKC-mediated TRAF1 induction, we pre-treated colon cancer cells with the MEK inhibitors PD98059 or U0126 prior to PMA treatment. Both of these agents significantly suppressed the stimulation of TRAF1 expression by PMA. In contrast, TRAF1 induction was not altered by the p38/MAPK inhibitor SB203580, the JNK inhibitor SP600125 or the PI3K inhibitor wortmannin. These results demonstrate the importance of ERK1/2 as an upstream effector of TRAF1 induction in colon cancers.

The transcriptional factor NF- κ B plays a crucial role in the regulation of numerous genes involved in the regulation of apoptosis (Karin and Lin, 2002). Activation of NF- κ B is mediated through phosphorylation, ubiquitination and subsequent degradation of the inhibitor I κ B, thus allowing the free NF- κ B dimer (p50/p65) to translocate to the nucleus and activate target genes (Karin and Ben-Neriah, 2000). The transcriptional activity of NF- κ B is also enhanced directly by phosphorylation at various sites on both p50 and p65 subunits; the kinases responsible for this phosphorylation include Ras, PKC and PKA (Mercurio and Manning, 1999; Karin and Ben-Neriah, 2000). TRAF1 expression is increased in certain cancer cells by cytokines of the TNF ligand family via NF- κ B activation (Wang *et al.*, 1998a), and also regulated by the β -catenin/NF- κ B pathway in human colon and breast cancers (Deng *et al.*, 2002). Consistent with these results, we found that the NF- κ B inhibitors abolished or significantly reduced PMA-induced TRAF1 expression, indicating that NF- κ B is also involved in TRAF1

activation by PMA. In this regard, analysis of the TRAF1 promoter has identified multiple κ B sites involved in TRAF1 upregulation (Schwenzer *et al.*, 1999). Specifically, the TRAF1 promoter contains three functional NF- κ B binding sites, κ B1, κ B3 and κ B5 (Schwenzer *et al.*, 1999). We identified the NF- κ B sites, κ B3 and κ B5, as critical for TRAF1 promoter induction by PMA in colon cancer cells. Furthermore, cotransfection with the I κ B super-repressor reduced basal TRAF1 promoter activity and blocked PMA-mediated promoter stimulation. Overexpression of IKK β , a major I κ B α kinase that results in degradation of I κ B α (Nakano *et al.*, 1998; Karin and Ben-Neriah, 2000), increased TRAF1 promoter activity whereas the kinase dead mutant, IKK β -KM, demonstrated a similar inhibition of TRAF1 promoter induction as noted by cotransfection of the I κ B super-repressor. Collectively, these findings confirm the importance of NF- κ B activation in downstream TRAF1 gene induction.

Our findings would suggest that the MEK/MAPK pathway contributes to NF- κ B activation and subsequent TRAF1 induction. Consistent with this notion, other investigators have reported NF- κ B activation mediated by MEK/MAPK, either acting dependently or independently of IKK activation. For example, Dhawan *et al.* (Dhawan and Richmond, 2002) found that, in melanoma cells, NF- κ B activity is dependent on both IKK and MEK/ERK activation. In other studies, ERK2 and ERK5 cooperatively regulate NF- κ B activity in NIH-3T3 cells (Pearson *et al.*, 2001), and the persistent activation of NF- κ B by IL-1 is mediated through the MEK/ERK pathway in rat vascular smooth muscle cells (Jiang *et al.*, 2002). Although our results clearly demonstrate the critical importance of NF- κ B activation and the contribution of the MEK/ERK pathway on TRAF1 induction in colon cancer cells, the precise details regarding whether these pathways act in concert or, alternatively, whether parallel pathways contribute to NF- κ B activation and, ultimately, TRAF1 induction are not known. Finally, the possibility that PMA is stimulating the synthesis of another mediator responsible for the crosstalk between the MEK/ERK and NF- κ B pathways cannot be ruled out.

In conclusion, we demonstrate selective TRAF1 induction in human colon cancer cells by PKC activation with PMA or bryostatin. Importantly, this induction occurs through a Ca²⁺-dependent PKC/Raf/ERK/NF- κ B-dependent pathway; activation of Ras does not appear to be required for TRAF1 induction. The potential functional role of selective TRAF1 induction in colon cancers remains to be clearly defined but could result in an increased growth advantage or increased chemoresistance in these tumors.

Materials and methods

Materials

PMA, wortmannin, actinomycin D and SB203580 were purchased from Sigma Chemical Company (St Louis, MD,

USA). Bis-indolylmaleimide (GF109203x), PD98059, Gö6983, G66976, rottlerin, MG132, PD98059, GGTI-286, -298, and -2133, FPT-I3 and gliotoxin were from Calbiochem (San Diego, CA, USA). The dual luciferase assay system and UO126 were from Promega (Madison, WI, USA). Bryostatin I and SP600125 was from BIOMOL (Plymouth Meeting, PA, USA). A23187 and ionomycin were from Alexis Biochemicals (San Diego, CA, USA). Antibodies against TRAF1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antiactin antibody was purchased from Sigma. The RPA III Ribonuclease Protection Assay System and the MAXI-Script SP6/T7 *in vitro* transcription kit were from Ambion (Austin, TX, USA). The human apoptosis template set (hAPO-5) was from BD Pharmingen (San Diego, CA, USA). [α -³²P] UTP (3000 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NY, USA). Total RNA was isolated using Ultraspec RNA Isolation System (Biotec Laboratories, Houston, TX, USA). Tissue culture media and reagents were obtained from GIBCO BRL (Grand Island, NY, USA). Polyvinylidene difluoride (PVDF) membranes for Western blots were from Millipore Corp (Bedford, MA, USA). Lipofectamine Plus reagent was purchased from Invitrogen (Carlsbad, CA, USA). The enhanced chemiluminescence (ECL) system for Western immunoblot analysis was purchased from Amersham.

Cell culture

The human colon cancer cell lines Caco-2, LoVo, DLD-1, SW480, SW620, CoLo201, CoLo205, HT29 and HCT116 were obtained from American Type Culture Collection (Rockville, MD, USA). Caco-2, LoVo and DLD-1 cells were incubated in MEM supplemented with either 15% (Caco-2) or 10% (LoVo, DLD-1, CoLo201, CoLo205) fetal calf serum (FCS), respectively. The human colon cancer cell line SW480 was grown in RPMI 1640/DMEM (1:3) supplemented with 10% FCS. Caco-2 cells with activated K-Ras were generous gifts from Dr E W. Gerner (Tucson, AZ, USA) (Taylor *et al.*, 2000), and K-Ras disrupted HCT116 and DLD-1 cells were generous gifts from Dr T Sasazuki (Fukuoka, Japan) (Okumura *et al.*, 1999); culture conditions for these cells were as described. PMA and inhibitors were initially dissolved in dimethyl sulfoxide (DMSO) and compared to cells treated with DMSO at the same final concentration.

RNA isolation and RNase protection assays

RNA was isolated from cells using Ultraspec RNA Isolation System according to the manufacturer's protocol. The RPA III Ribonuclease Protection Assay Kit (Ambion) was used for the detection of multiple, specific mRNA species following the manufacturer's instructions and as we have previously described (Wang *et al.*, 2002). ³²P-labeled antisense RNA probes were prepared using the Human Apoptosis hAPO-5 Template Set (BD Pharmingen) and MAXIscript SP6/T7 *In vitro* Transcription Kit (Ambion), according to the manufacturer's protocol. Samples were analysed by electrophoresis on a 5% denaturing polyacrylamide gel and detected by autoradiography.

Protein preparation and Western immunoblot

Western immunoblot analyses were performed as described previously (Wang *et al.*, 2002). Cells were lysed with TNN buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.5 mM Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and 25 μ g/ml each of aprotinin, leupeptin and

pepstatin A) at 4°C for 30 min. Lysates were clarified by centrifugation (10 000 *g* for 30 min at 4°C) and protein concentrations determined, as described by Bradford (Bradford, 1976). Briefly, total protein (100 µg) was resolved on a 10% polyacrylamide gel and transferred to PVDF membranes. Filters were incubated overnight at 4°C in blotting solution (Tris-buffer saline containing 5% nonfat dried milk and 0.1% Tween 20). TRAF1 and actin were detected with specific antibodies to TRAF1 and β-actin after blotting with a horseradish peroxidase-conjugated secondary antibody and visualized using ECL detection.

Transient transfection, luciferase assays

The TRAF1 promoter-reporter constructs and their site-directed mutants were generous gifts from Dr Harold Wajant (University of Stuttgart, Germany) (Schwenzer *et al.*, 1999). The expression vector encoding the phosphorylation-defective IκBα(S32A/S36A), IκBαM, was a generous gift from Dr Allan R Brasier (University of Texas Medical Branch, Galveston, TX, USA) (Brasier *et al.*, 2001). The expression vectors for IKKβ and IKKβ-KM (K44A) were generous gifts

from Dr Hiroyasu Nakano (Juntendo University, Tokyo, Japan) (Nakano *et al.*, 1998). Raf-C4, Raf-C4M17, Ras-N17 and pZIP-neo(x) plasmids were described previously (Evers *et al.*, 1995). SW480 cells were seeded in 24-well plates (0.3 × 10⁶ cells/well) 1 day prior to transfection. Cells were transfected with 1 µg of TRAF1 promoter plasmids with or without the expression plasmids or empty vectors and 20 ng of pRL-TK (internal control) per well using Lipofectamine Plus reagent. Approximately 40 h after transfection, cells were treated with a variety of reagents and harvested. Luciferase activities were assayed with the dual luciferase assay system as we have previously described (Wang *et al.*, 2001).

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