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Protein phosphatase 2A is a critical regulator of protein kinase C ζ signaling targeted by SV40 small t to promote cell growth and NF-κB activation

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We have reported that inhibition of protein phosphatase 2A (PP2A) by expression of SV40 small t stimulates the mitogenic MAP kinase cascade. Here, we show that SV40 small t can substitute for tumor necrosis factor-α (TNF-α) or serum and stimulate atypical protein kinase C ζ (PKC ζ) activity, resulting in MEK activation, cell proliferation and NF-κB-dependent gene transcriptional activation in CV-1 and NIH 3T3 cells. These effects were abrogated by co-expression of kinase-deficient PKC ζ and inhibition of phosphatidylinositol 3-kinase p85α-p110 by wortmannin, LY294002 and a dominant-negative mutant of p85α. In contrast, expression of kinase-inactive ERK2 inhibited small t-dependent cell growth but was unable to abolish small t-induced NF-κB transactivation. Our results provide the first *in vivo* evidence for a critical regulatory role of PP2A in bifunctional PKC ζ signaling pathways controlled by phosphatidylinositol 3-kinase. Constitutive activation of PKC ζ and NF-κB following inhibition of PP2A supports new mechanisms by which SV40 small t promotes cell growth and transformation. By establishing PP2A as a key player in the response of cells to growth factors and stress signals like TNF-α, our findings could explain why PP2A is a primary target utilized during SV40 infection to alter cellular behavior.

Keywords: NF-κB/PI 3-kinase/PKC ζ/PP2A/SV40 small t

Introduction

The dynamic process of signal transduction involves the concerted action of both protein kinases and protein phosphatases. Protein phosphatase 2A (PP2A), a major protein serine/threonine phosphatase in most mammalian tissues, is implicated in the regulation of various cellular processes, such as cell growth and transformation (reviewed in Mumby and Walter, 1993). Interestingly, PP2A is a target for proteins expressed by DNA tumor viruses, including simian virus 40 (SV40) small t antigen (small t). During SV40 infection, intracellular PP2A becomes complexed to small t (Rundell, 1987). Association of small t with PP2A results in specific inhibition of PP2A activity towards several substrates *in vitro* (Yang *et al.*, 1991). Small t is required for efficient transformation of several cell types by SV40, especially when assayed in growth-arrested cells (Martin *et al.*, 1979). Small t also stimulates viral DNA replication in the permissive monkey CV-1 cells (Cicala *et al.*, 1994). We have shown that expression of small t in quiescent CV-1 cells stimulates the MAP kinase pathway and promotes cell growth, through specific inhibition of PP2A (Sontag *et al.*, 1993). Small t also activates AP-1 in microinjected CV-1 cells in a MAP kinase-dependent fashion (Frost *et al.*, 1994). However, the mechanisms supporting the role of small t during SV40 infection are far from being completely understood.

Like PP2A, the atypical calcium-independent protein kinase C ζ isoform (PKC ζ) has been involved in the control of mitogenic signal transduction and survival (Berra *et al.*, 1993, 1995; Gomez *et al.*, 1995; Diaz-Meco *et al.*, 1996; Kieser *et al.*, 1996; Powell *et al.*, 1996). PKC ζ also plays a pivotal role in tumor necrosis factor-α (TNF-α) activation of NF-κB (Diaz-Meco *et al.*, 1993, 1994; Dominguez *et al.*, 1993; Lozano *et al.*, 1994), an inducible transcriptional activator that participates in the control of cell proliferation and survival, as well as in inflammatory response and viral gene expression (reviewed in Bauerle and Baltimore, 1996). NF-κB has been especially implicated in the transcriptional activation of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (reviewed in Gaynor, 1992). NF-κB is the prototype of a family of heterodimeric transcription factors composed of monomers that bind to DNA and the I-κB inhibitors (reviewed in Baldwin, 1996). NF-κB is retained in the cytoplasm in its inactive form by the I-κB α inhibitor. Upon stimulation of cells, I-κB α becomes phosphorylated and dissociates from NF-κB, resulting in the translocation of NF-κB to the nucleus, where it carries out its transactivation function. The rapid phosphorylation of I-κB α represents a possible signal for its proteolysis (Brown *et al.*, 1995).

PKC ζ activity can be stimulated by phosphatidylinositol 3,4,5-trisphosphate (PIP3) *in vitro*, suggesting that PKC ζ could be activated by phosphoinositide 3-kinase (PI 3-kinase) *in vivo* (Nakanishi *et al.*, 1993). PI 3-kinase is a heterodimer composed of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (P110), each of which exists in at least two isoforms (α and β). This lipid kinase participates in a variety of cellular functions, including mitogenic signaling, cell transformation, inhibition of apoptosis, intracellular vesicle trafficking and secretion, and regulation of actin and integrin functions (reviewed recently in Carpenter and Cantley, 1996).

To approach the mechanism whereby small t regulates the MAP kinase cascade and cell proliferation, we examined its effects on PKC ζ activity in transfected CV-1 and NIH 3T3 cells. Our results demonstrate that

expression of small t stimulates PKC ζ activity in quiescent cells, which leads to induction of NF-κB-controlled gene expression and cell growth. Data are shown here demonstrating that all the cellular effects of small t can be suppressed by co-expression of dominant-negative mutant forms of PKC ζ and PI 3-kinase. Our results establish for the first time that PP2A is critically involved in the regulation of PI 3-kinase/PKC ζ signaling. They also identify new mechanisms by which constitutive activation of this mitogenic signaling by deregulation of PP2A contributes to the transformation- and proliferation-enhancing activities of small t.

Results

Expression of small t induces PKC ζ activation

We have reported that interaction of small t with PP2A stimulates the activity of the MAP kinase kinase MEK1 in CV-1 cells (Sontag *et al.*, 1993). However, small t does not affect the activity of Raf-1, an upstream activator of MEK1. Recent studies have shown that MEK can be phosphorylated by PKC ζ *in vitro* and in Cos cells (Diaz-Meco *et al.*, 1994; Berra *et al.*, 1995), raising the possibility that PKC ζ is a target for small t. To address this hypothesis, PKC ζ activity was first determined in immunoprecipitates prepared from control and small t-transfected CV-1 cells, using $MBP_{4–14}$ or recombinant MEK1 as a substrate (Figure 1). Equivalent amounts of PKC ζ were recovered in each immunoprecipitate (Figure 1A). Although $MBP_{4–14}$ was found to be a better PKC ζ substrate than MEK1 (not shown), comparable relative kinase activities were obtained with either substrate. Control experiments were performed to verify that phosphorylation of $MBP_{4–14}$ and MEK1 resulted from PKC ζ. Phosphorylation was completely abolished when immunoprecipitation was carried out in the presence of competing PKC ζ peptide, or when the kinase assays were performed in the presence of a PKC ζ inhibitor peptide (not shown), as reported previously (Diaz-Meco *et al.*, 1994).

Incubation of quiescent CV-1 cells with serum stimulated the basal level of PKC ζ activity by ~3.5-fold. Expression of small t alone induced an ~3.3-fold stimulation of PKC ζ activity in serum-starved cells. The kinase activity was enhanced further following serum stimulation of small t-expressing cells. The extent of small t-induced PKC ζ activation was comparable with that observed when cells were incubated with okadaic acid, a potent inhibitor of protein phosphatase 1 and PP2A. Okadaic acid caused an ~3.4- and ~5.4-fold increase in kinase activity in unstimulated or serum-stimulated control cells, respectively. As shown on Figure 1C, similar results were obtained when cells were treated with TNF-α instead of serum. Not only were small t or okadaic acid as potent as TNF-α in inducing PKC ζ activation, but they also cooperated with TNF-α to stimulate the kinase. Further evidence for the involvement of PP2A in small t-induced PKC ζ activation was obtained using transfection with well-characterized small t mutants (Sontag *et al.*, 1993). Expression of small t mutant 8, which inhibits PP2A nearly as efficiently as wild-type small t, strongly stimulated PKC ζ. In contrast, small t mutant 3, deficient in inhibiting PP2A, did not alter PKC ζ activity (Figure 1B and C).

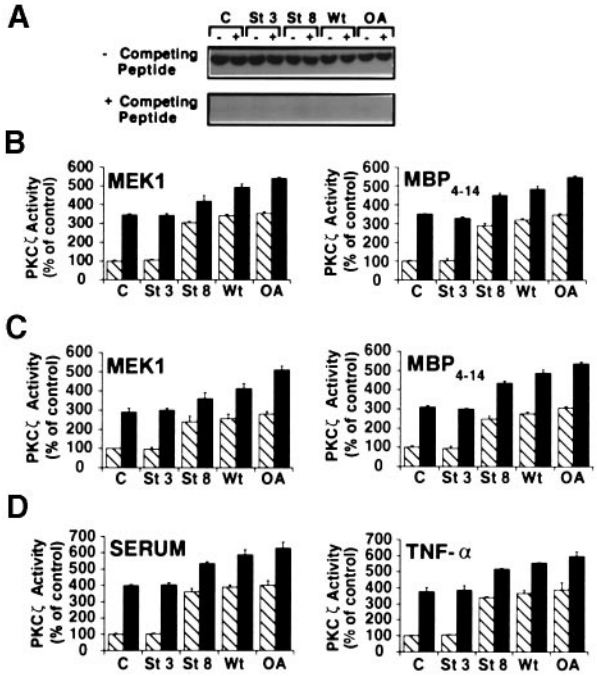

Fig. 1. Inhibition of PP2A induces PKC ζ activation. Control non-transfected cells (C) or cells transfected with 15 μg each of pCMV5 encoding wild-type small t (Wt), small t mutant 3 (St 3) or small t mutant 8 (St 8) were serum starved then either left untreated [(–), hatched bars] or stimulated for 10 min with serum or TNF-α [(+), closed bars]. Control quiescent cells were also incubated for 1 h with 100 nM okadaic acid (OA) prior to stimulation. (**A**) Representative Western blot analysis of PKC ζ in CV-1 immunoprecipitates carried out either in the absence or presence of competing PKC ζ peptide. (**B**) PKC ζ activity was assayed in immunoprecipitates from unstimulated and serum-stimulated CV-1 cells using MEK1 or $MBP_{4–14}$ as a substrate. (**C**) Same as (B), except that PKC ζ activity was assayed in immunoprecipitates from unstimulated and TNF-α-stimulated CV-1 cells. (**D**) PKC ζ activity was assayed in immunoprecipitates from unstimulated and serum- or TNF-α-stimulated NIH 3T3 cells using MEK1 as a substrate. For (B), (C) and (D), PKC ζ activity is expressed as the percentage of the control activity measured in immunoprecipitates from unstimulated, non-transfected cells. Values represent the mean ± SD of duplicate determinations from three separate experiments. The basal activity of PKC ζ in control cells was not affected by transfection with pCMV5 alone (not shown).

Figure 1D shows that comparable findings were obtained in NIH 3T3 fibroblasts.

Together, these results indicate that small t can substitute for serum or TNF-α and activate endogenous PKC ζ in CV-1 and NIH 3T3 cells. Moreover, small t potentiates the stimulatory effects of serum or TNF-α on PKC ζ activity. Data obtained with small t mutants support the hypothesis that inhibition of PP2A is sufficient to induce PKC ζ activation significantly in CV-1 and NIH 3T3 cells.

PKC ζ is critical for small t-dependent MEK activation and cell proliferation

Since PKC ζ was strongly activated in small t-expressing cells (Figure 1), transfection experiments were performed to determine whether small t-dependent MEK stimulation was mediated by PKC ζ. CV-1 cells expressing wild-type or mutant small t proteins were transfected with plasmids encoding a dominant-negative form of PKC ζ (Berra *et al.*, 1993) or the corresponding empty vector, then serum starved. Phosphorylation of endogenous MEK was then

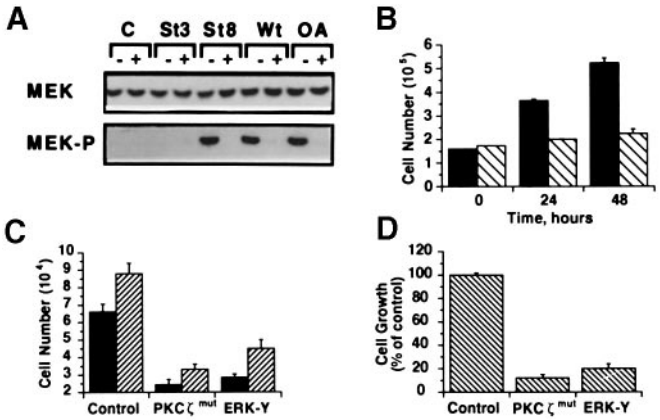

Fig. 2. Co-expression of a kinase-inactive mutant of PKC ζ blocks small t-dependent MEK phosphorylation and cell proliferation. (**A**) CV-1 cells were co-transfected with 5 μg of either pCMV5 (C), or pCMV5 encoding wild-type small t (Wt), small t mutant 3 (St3) or small t mutant 8 (St8), in combination with 10 μg of either pRcCMV (–) or pRcCMV-ζPKC^{mut} (+) encoding kinase-deficient PKC ζ. Cells were then serum starved and harvested for Western blot analysis. Quiescent CV-1 cells were also incubated for 1 h with 100 nM okadaic acid (OA) prior to lysis. Immunoblots were analysed for the presence of MEK or phosphorylated MEK (MEK-P) using phosphorylation-independent and phospho-specific antibodies, respectively. Shown here are representative results from one of three experiments. (**B**) CV-1 cells stably expressing wild-type small t were transfected with 15 μg of pRcCMV (closed bars) or pRcCMV-ζPKC^{mut} (hatched bars). Cells were seeded 24 h post-transfection at a density of 1.7×10^5 cells per 100 mm dish and incubated in DMEM containing 0.5% serum. Cell growth was determined by counting cells every 24 h. Small t-expressing cells cultured for 48 h in the presence of 10% serum grew to a density of 6×10^5 cells per 100 mm dish (not shown). (**C**) CV-1 cells stably expressing wild-type small t were non-transfected (Control) or transfected with 15 μg of pRcCMV-ζPKC^{mut} (PKCζ^{mut}) or pCMV5-ERK2-Y185F encoding kinase-inactive ERK2 (ERK-Y). Cells were seeded 24 h post-transfection at a density of 2×10^4 cells per 60 mm culture dish and incubated in DMEM containing 0.5% (closed bars) or 10% serum (hatched bars). Cell growth was determined after 48 h. (**D**) NIH 3T3 were transfected with 5 μg of pCMV5 wild-type small t in combination with 10 μg each of pCMV5 (Control), pRcCMV-ζPKC^{mut} (PKCζ^{mut}) or pCMV5-ERK2-Y185F (ERK-Y), replated 24 h post-transfection and incubated for 48 h in DMEM containing 0.5% serum. Results are expressed as the percentage of cell growth determined in control small t-expressing cells. For (B), (C) and (D), values shown are the mean ± SD of triplicate determinations from three separate experiments. Transfection with the empty vectors pRcCMV or pCMV5 did not affect cell growth (not shown).

determined in total cell extracts as a biological read-out for its activation (Figure 2A). Immunoblotting with a phosphorylation-independent antibody revealed that identical amounts of MEK proteins were expressed in each cell fraction. Consistent with small t-induced MEK activation (Sontag *et al*., 1993), a significant phosphorylation of endogenous MEK was detected with a phospho-specific MEK antibody in serum-starved, small t-expressing cells. A comparable MEK phosphorylation state was observed in cells transfected with small t mutant 8, or treated with okadaic acid. In contrast, MEK was predominantly in its unphosphorylated form in quiescent control cells or in cells transfected with small t mutant 3. Expression of the PKC ζ mutant dramatically prevented small t- or okadaic acid-induced MEK phosphorylation, as judged by the disappearance of the signal for phosphorylated MEK.

Since small t promotes the growth of quiescent CV-1 cells mostly through activation of the MAP kinase cascade (Sontag *et al.*, 1993), the above data suggested that PKC ζ could be involved directly in the proliferative effects of small t. To verify this hypothesis, CV-1 cells stably expressing small t were transfected with the plasmid encoding kinase-inactive PKC ζ or the corresponding empty construct, then cultured for 48 h in medium containing 0.5% serum. Figure 2B shows that CV-1 cells stably expressing small t proliferated well under these poor conditions, and grew to densities almost as high as a subset of control cells cultured with 10% serum, as observed previously in transiently transfected cells (Sontag *et al.*, 1993). In contrast, the growth rate of cells co-expressing the dominant-negative form of PKC ζ was substantially decreased. Kinase-deficient PKC ζ produced an ~91% growth inhibition of serum-starved cells and was more potent than kinase-inactive ERK2 in blocking cell proliferation (Figure 2C). This inhibitory effect was less pronounced when cells were cultured in the presence of 10% serum, as reported previously with kinase-deficient ERK2 (Sontag *et al.*, 1993). As observed in CV-1 cells, overexpression of kinase-inactive ERK2 and PKC ζ prevented small t-stimulated proliferation of serum-starved NIH 3T3 cells (Figure 2D).

Together, these results suggest that small t-induced activation of PKC ζ primarily accounts for the ability of small t to stimulate the MAP kinase cascade and promote serum-independent cell growth.

Small t promotes the degradation of cytoplasmic I-κB α and activates NF-κB-dependent gene expression

TNF-α-mediated stimulation of PKC ζ results in I-κB α degradation (Diaz-Meco *et al.*, 1994) and NF-κB-dependent promoter activation (Diaz-Meco *et al.*, 1993; Dominguez *et al.*, 1993; Lozano *et al.*, 1994). If small t-mediated PKC ζ activation was physiologically relevant, it should produce similar effects. The distribution of I-κB α in small t-transfected CV-1 cells was examined by immunoblot analysis. Figure 3A shows that the amounts of cytoplasmic I-κB α were largely decreased in small t-expressing cells relative to control cells. To verify that these differences did not result from discrepancies in the amounts of proteins loaded on the gel, the same blot was reprobed with an antibody recognizing the p110 precursor of NF-κB. In contrast to I-κB α, the p110 protein had a similar pattern of expression in the cytoplasm of cells either transfected or not with small t. Thus, the disappearance of the signal for I-κB α suggests that inhibition of PP2A by small t provokes the degradation of cytoplasmic I-κB α. This rapid proteolytic turnover of I-κB α represents a likely consequence of the constitutive activation of PKC ζ by small t.

We then examined whether small t was able to induce functional NF-κB-driven gene expression by performing reporter gene assays. CV-1 cells were transfected with plasmids encoding wild-type or mutant small t proteins, together with NF-κB-dependent luciferase reporter plasmids. Luciferase activity was measured in total extracts prepared from cells that had been serum starved, then either left untreated or stimulated with TNF-α. As shown in Figure 3B, incubation of control cells with TNF-α produced an ~8-fold activation of the κB-ConA-luc lucifer-

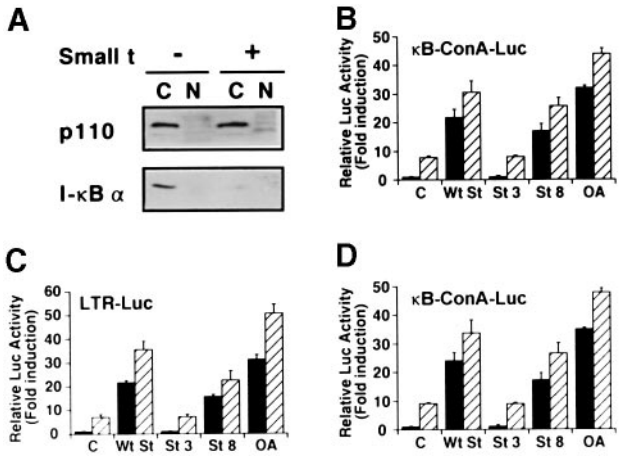

Fig. 3. Inhibition of PP2A induces I-κB α degradation and NF-κB-mediated gene transcriptional activation. (**A**) Western blot analysis of I-κB α and NF-κB p110 precursor protein levels in cytosolic (C) and nuclear (N) fractions from control (–) or small t-expressing (+) CV-1 cell extracts. (**B**) CV-1 cells were transfected with the κB-ConA-luc luciferase reporter construct (5 μg) plus 10 μg of either pCMV5 (C), or pCMV5 encoding wild-type small t (Wt St), small t mutant 3 (St 3) or small t mutant 8 (St 8). Cells were serum starved and either left untreated (closed bars) or stimulated for 4 h with TNF-α (hatched bars). Cells transfected with the luciferase construct alone were also incubated for 3 h with 100 nM okadaic acid (OA) prior to stimulation. The cells were then lysed and analyzed for luciferase activity. Data are expressed as fold induction of the normalized luciferase (Luc) activity measured in unstimulated control cells transfected with the luciferase reporter construct alone. (**C**) Same as (B), except that CV-1 cells were transfected with the HIV-1 LTR-luc luciferase reporter construct instead of κB-ConA-luc. (**D**) NIH 3T3 were transfected and processed as described in (B) for CV-1 cells. For (B), (C) and (D), values shown are the mean ± SD of duplicate assays from three separate experiments.

ase reporter construct, which harbors three copies of the NF-κB enhancer from HIV-1 LTR. Co-expression of wild-type small t or small t mutant 8, but not mutant 3, resulted in an ~22- and ~17-fold stimulation of luciferase activity, respectively. Likewise, okadaic acid promoted an ~32-fold up-regulation of the reporter gene activity in unstimulated control cells. As observed with okadaic acid, small t synergistically cooperated with TNF-α in strongly inducing κB-ConA-luc. Parallel control experiments showed that only negligible levels of luciferase activity were detected in cells transfected with the ConA-luc vector deleted from the NF-κB enhancer sites (not shown).

Further evidence that NF-κB induced upon small t expression was transcriptionally functional came from assays measuring the activity of LTR-luc (Figure 3C), a luciferase reporter construct under the control of the HIV-1 LTR, which contains two essential NF-κB-binding sites. Expression of wild-type small t stimulated NF-κB-mediated HIV-1 LTR transcription by ~21-fold relative to unstimulated control cells (Figure 3C). A comparable up-regulation was measured in cells transfected with small t mutant 8, but not with small t mutant 3. Incubation of control cells with okadaic acid also produced an ~31-fold transcriptional activation of HIV-1 LTR. The induction of LTR-luc activity by small t or okadaic acid was considerably enhanced by further stimulation of the cells with TNF-α.

Additional experiments revealed that small t induced similar transactivating effects in NIH 3T3 fibroblasts (Figure 3D). Taken together, these results suggest that inhibition of PP2A activity provokes the degradation of I-κB α and NF-κB-dependent gene transcriptional activation.

Small t-induced activation of NF-κB-dependent gene expression is dependent on PKC ζ, but not on ERK2

The mechanisms governing small t-mediated induction of NF-κB-controlled promoters were assessed by examining the effects of deregulating PKC ζ and ERK2 on this activation. Expression of the dominant-negative form of PKC ζ has been shown to suppress NF-κB-dependent gene transcription in NIH 3T3 cells (Lozano *et al.*, 1994), Cos cells (Berra *et al.*, 1995) and U937 cells (Folgueira *et al.*, 1996). Likewise, expression of this PKC ζ mutant in CV-1 cells fully compromised the ability of small t or okadaic acid to transactivate κB-ConA-luc (Figure 4A) or HIV-1 LTR-luc (not shown). In contrast, expression of kinase-deficient ERK2 did not inhibit the induction of κB-ConA-luc activity by either small t or TNF-α (Figure 4B). Furthermore, overexpression of wild-type ERK2 did not affect the levels of luciferase activity determined in control or small t-transfected cells (not shown). These data suggest that ERK2 is not directly involved in activation of NF-κB-dependent gene expression. Thus, in CV-1 cells, induction of NF-κB-controlled gene transcription by small t is mediated by activation of PKC ζ, independently of ERK2. Similar conclusions were reached when experiments were performed in NIH 3T3 cells (Figure 4C).

Inhibition of PI 3-kinase suppresses small t-induced stimulation of PKC ζ

It has been proposed that PI 3-kinase acts as an upstream regulator of PKC ζ (Nakanishi *et al.*, 1993). To address whether PI 3-kinase was modulating small t-induced PKC ζ activation, PKC ζ activity was measured in small t-expressing cells that had been serum starved and incubated with increasing concentrations of wortmannin (Yano *et al.*, 1993) or LY294002 (Vlahos *et al.*, 1994), two potent inhibitors of PI 3-kinase. Equivalent amounts of PKC ζ were recovered in immunoprecipitates from wortmannin- or LY294002-treated or untreated cells (not shown). Figure 5A shows that wortmannin and LY294002 inhibited small t-induced PKC ζ activation in a concentration-dependent manner. A maximal ~87 and ~85% inhibitory effect was observed with 100–500 nM of wortmannin and 100 μM of LY294002, respectively. Higher drug concentrations were found to have a slightly toxic effect on cells exposed to them over a long period of time, and were thus avoided. Reduction of PKC ζ activity was not due to an indirect effect of the PI 3-kinase inhibitors on small t, since the amounts of expressed small t proteins were not affected by treatment with these drugs (not shown).

Further demonstration of PI 3-kinase involvement in small t-induced PKC ζ activation was obtained in experiments where small t-expressing cells were co-transfected with SRα-Δp85, a plasmid encoding Δp85, a dominant-negative mutant form of PI 3-kinase p85α regulatory subunit. Transfection with SRα-Δp85 has been shown to compromise PI 3-kinase activation by insulin and other agonists (Hara *et al.*, 1994). Expressed Δp85 mimicked

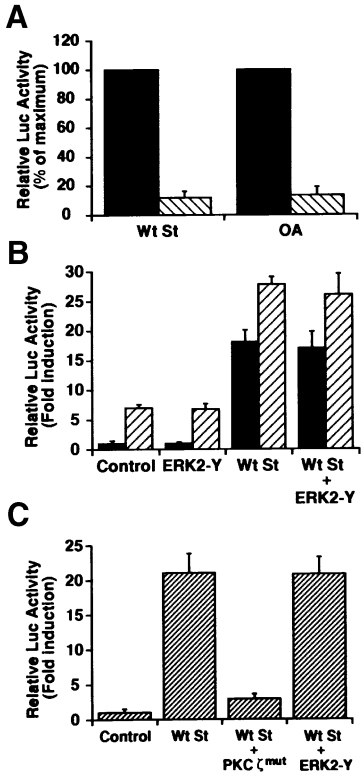

Fig. 4. Small t activates NF-κB-dependent gene transcription through stimulation of PKC ζ, but not ERK2. (**A**) Control CV-1 cells or CV-1 cells expressing small t (Wt St) were transfected with 5 μg of κB-ConA-luc plus 10 μg of pRcCMV alone (closed bars) or pRcCMV-ζPKC^{mut} (hatched bars), then serum starved. Quiescent control cells were incubated for 3 h with 100 nM okadaic acid (OA) before being harvested for luciferase assays. Results are expressed as the percentage of the maximal normalized luciferase (Luc) activity measured in each subset of cells transfected with the luciferase reporter construct alone. (**B**) CV-1 cells were transfected with 5 μg of κB-ConA-luc plus 10 μg of pCMV5 (Control) or pCMV5-ERK2-Y185F (ERK2-Y). CV-1 cells expressing small t were also co-transfected with 5 μg of κB-ConA-luc plus 10 μg of pCMV5 (Wt St) or pCMV5-ERK2-Y185F (Wt St + ERK2-Y). The cells were then serum starved and left untreated (closed bars) or stimulated for 4 h with TNF-α (hatched bars). Results are expressed as fold induction of the normalized luciferase (Luc) activity measured in unstimulated control cells. (**C**) NIH 3T3 cells were transfected with κB-ConA-luc (3 μg) and the following plasmids: 12 μg of pCMV5 (Control), and 6 μg each of either pCMV5 wild-type small t plus pCMV5 (Wt St), pCMV5 wild-type small t plus pRcCMV-ζPKC^{mut} (Wt St + PKCζ^{mut}) or pCMV5 wild-type small t plus pCMV5-ERK2-Y185F (Wt St + ERK2-Y). The cells were then serum starved and analysed for luciferase activity. Results are expressed as fold induction of the normalized luciferase (Luc) activity measured in unstimulated control cells. For all panels, values shown are the mean ± SD of duplicate assays from three separate experiments.

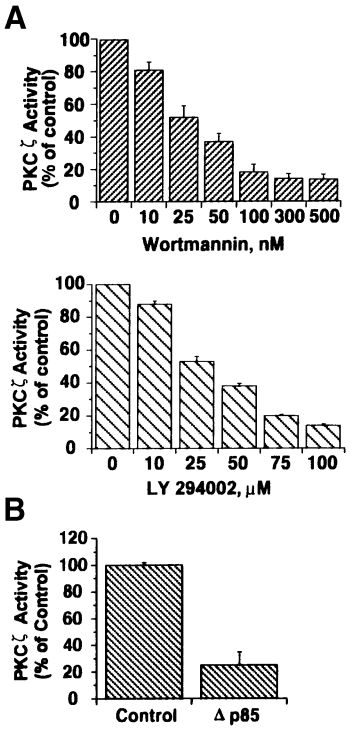

Fig. 5. Inhibition of PI 3-kinase abrogates small t-induced activation of PKC ζ. (**A**) CV-1 cells expressing wild-type small t were serum starved then treated for 5 h with the indicated concentrations of wortmannin (upper panel) or LY294002 (lower panel). Values shown are expressed as the percentage of the control PKC ζ activity measured in immunoprecipitates from untreated small t-transfected cells, and represent the mean ± SD of duplicate determinations from three separate experiments. (**B**) CV-1 cells expressing wild-type small t were transfected with 15 μg of SRα-Δp85 (Δp85) encoding a dominant-negative mutant of PI 3-kinase p85 subunit, or the control vector alone (Control), and processed as in (A).

the effects of wortmannin and LY294002, and substantially inhibited small t-dependent PKC ζ activation (Figure 5B).

PI 3-kinase controls small t-induced cell growth and NF-κB-dependent gene transactivation

The above results prompted us to examine whether a PI 3-kinase-sensitive pathway was implicated in the proliferative effects of small t. Figure 6A shows that addition of 100 nM wortmannin suppressed small t-dependent cell growth. LY294002 also inhibited small t-dependent cell proliferation in a concentration-dependent manner (Figure 6B). Growth inhibition was not due to possible toxic effects of these drugs, since cell growth could be resumed normally when they were removed from the cell medium (not shown). As observed with the PI 3-kinase inhibitors, expression of Δp85 completely blocked small t-induced proliferation in CV-1 cells (Figure 6C) and NIH 3T3 fibroblasts (Figure 6D). In contrast to cells cultured in medium containing 0.5% serum, wortmannin (Figure 6A), LY294002 (not shown) or Δp85 (not shown) had no marked effects on the growth response of cells exposed to 10% serum.

These findings suggested that PI 3-kinase may also participate directly in the regulation of NF-kB-controlled promoter activation by PP2A. Consistent with this hypothesis, incubation of quiescent CV-1 cells with wortmannin or LY294002 abolished both small t- and okadaic acid-mediated transactivation of κB-ConA-luc (Figure 7A). In addition, expression of Δp85α completely prevented small t-induced κB-ConA-luc up-regulation in CV-1 (Figure 7B) and NIH 3T3 cells (Figure 7C). Wortmannin, LY294002 and Δp85α also suppressed the ability of small t and okadaic acid to potentiate the effects of TNF-α on NF-κB-dependent promoter activation (not shown).

Together, these results strongly support the hypothesis that PI 3-kinase p85α-p110 regulates small t-mediated stimulation of NF-κB-dependent gene expression and cell growth.

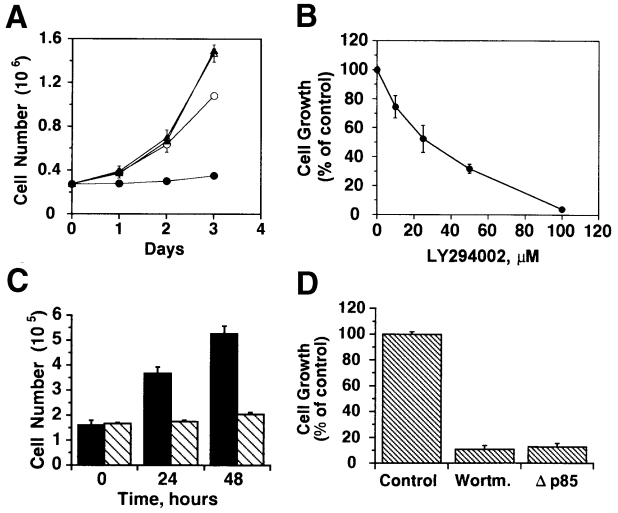

Fig. 6. Inhibition of PI 3-kinase activity suppresses small t-dependent cell proliferation. (**A**) CV-1 cells expressing small t were seeded at a density of 2.8×10^5 cells per 100 mm dish and incubated in DMEM containing either 0.5% (circles) or 10% serum (triangles), in the absence (open symbols) or presence (closed symbols) of 100 nM wortmannin. Cell growth was determined by counting cells every other day. (**B**) CV-1 cells expressing small t were cultured for 48 h in DMEM containing 0.5% serum in the presence of 0–100 μM LY294002. Results are expressed as the percentage of cell growth determined in the absence of LY294002. (**C**) CV-1 cells expressing small t were non-transfected (closed bars) or transfected with 15 μg of SRα-Δp85 (hatched bars). Cells were seeded 24 h post-transfection at a density of ~1.6×10^5 cells per 100 mm culture dish and incubated in DMEM containing 0.5% serum. Cell growth was determined by counting cells every 24 h. (**D**) NIH 3T3 cells were transfected with pCMV5 wild-type small t (5 μg) in combination with 10 μg of SRα-Δp85 (Δp85) or the corresponding empty vector (Control), replated 24 h post-transfection and incubated for 48 h in DMEM containing 0.5% serum. One set of control small t-expressing cells was also cultured in the presence of 100 nM wortmannin (Wortm.). Results are expressed as the percentage of cell growth determined in control, untreated small t-expressing cells. For all panels, values represent the mean ± SD of triplicate determinations from three separate experiments.

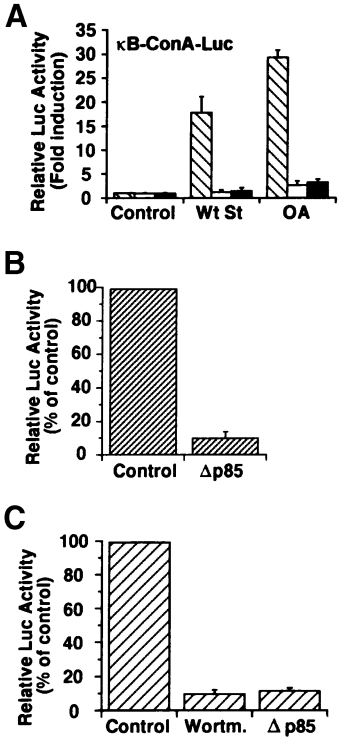

Fig. 7. Inhibition of PI 3-kinase activity suppresses small t-dependent NF-κB-dependent gene transcriptional activation. (**A**) CV-1 cells were co-transfected with 5 μg of κB-ConA-luc and 10 μg of pCMV5 (Control) or pCMV5-small t (Wt St). The transfected cells were serum starved and incubated for 5 h in the absence (hatched bars) or presence of 100 nM wortmannin (open bars) or 75 μM LY294002 (closed bars), before being harvested for luciferase assays. Control quiescent cells were also incubated for 3 h with 100 nM okadaic acid (OA). Results are expressed as fold induction of the mean normalized luciferase (Luc) activity measured in unstimulated control cell extracts. (**B**) CV-1 cells expressing small t were co-transfected with κB-ConA-luc (5 μg) plus 10 μg of SRα-Δp85 (Δp85) or the corresponding empty vector (Control), then serum starved. Data are expressed as the percentage of the mean normalized luciferase (Luc) activity measured in extracts from control small t-expressing cells. (**C**) NIH 3T3 cells were co-transfected with κB-ConA-luc (3 μg) and pCMV5 wild-type small t (6 μg), plus 6 μg each of SRα-Δp85 (Δp85) or the corresponding empty vector (Control), then serum-starved. One set of control small t-expressing cells was incubated for 5 h with 100 nM wortmannin (Wortm.). Results are expressed as the percentage of the mean normalized luciferase (Luc) activity measured in extracts from control, untreated small t-expressing cells. For all panels, values shown are the mean ± SD of duplicate determinations from three separate assays.

Discussion

This study builds upon our previous finding that specific inhibition of PP2A by transient expression of SV40 small t in CV-1 cells results in MEK and ERK2 activation, subsequent stimulation of AP-1 activity (Frost *et al.*, 1994) and cell proliferation (Sontag *et al.*, 1993). Here, we first provide evidence that interaction of small t with PP2A induces the constitutive activation of the MAP kinase cascade through stimulation of atypical PKC ζ activity (Figures 1 and 2), in agreement with previous reports suggesting that PKC ζ is involved in ERK activation (Berra *et al.*, 1995), cell growth control (Berra *et al.*, 1993) and AP-1-dependent promoter transactivation (Bjorkoy *et al.*, 1995). Although we chose to examine primarily the function of small t in monkey kidney CV-1 cells because those cells represent a physiologically relevant model for studying the function of SV40 proteins, we found that small t exerts comparable biological effects in mouse NIH 3T3 fibroblasts (Figures 1–7) and human neuronal precursor NT2 cells (not shown).

PKC isozymes are dually regulated by a complex succession of phosphorylation and lipid messengers (reviewed in Newton, 1997). PP2A is capable of dephosphorylating all the PKCs at two conserved sites, without affecting their activity (Keranen *et al.*, 1995). The inability of PP2A to inactivate PKCs directly suggests that inhibition of PP2A by small t does not stimulate PKC ζ activity directly, but rather by modulating the activity of an upstream regulator. Small t-induced PKC ζ activation was potently inhibited (Figure 5A) by two structurally unrelated compounds, wortmannin and LY294002, which inhibit PI 3-kinase activity by different mechanisms (Yano *et al.*, 1993; Vlahos *et al.*, 1994). Overexpression of a mutant of the p85α regulatory subunit of PI 3-kinase (Hara *et al.*, 1994) was as efficient as these inhibitors in blocking PKC ζ activation (Figure 5B). Thus, PI 3-kinase p85α-p110 emerges as an upstream activator of PKC ζ. This conclusion is supported by the finding that PKC ζ activity can be stimulated very efficiently *in vitro* by physiological

concentrations of PIP3, a product of PI 3-kinase (Nakanishi *et al.*, 1993). In addition, PI 3-kinase has been implicated in cell growth regulation (Roche *et al.*, 1994) and MEK activation in other cellular systems (Karnitz *et al.*, 1995). Like PKC ζ, atypical PKC λ functions downstream of PI 3-kinase and activates AP-1 in response to growth factor stimulation (Akimoto *et al.*, 1996). PI 3-kinase also regulates platelet-derived growth factor (PDGF)-dependent activation of PKC ε in HepG2 cells (Moriya *et al.*, 1996). Thus, PI 3-kinase controls the activation of multiple PKC isoforms that function in redundant and independent pathways. The putative regulatory role of PP2A in these pathways remains to be elucidated. Beside PI 3-kinase, multiple intracellular components interacting with and/or modulating PKC ζ activity have been identified. One of them is ceramide, which is released after TNF-α-induced sphingomyelin hydrolysis (reviewed in Hannun, 1996) and directly binds to and activates PKC ζ *in vitro* and *in vivo* (Lozano *et al.*, 1994; Müller *et al.*, 1995). A ceramide-activated protein phosphatase (CAPP), which shares several properties with PP2A, is implicated in some of the cellular activities of ceramide (reviewed in Hannun, 1996). It will be interesting in the future to investigate whether CAPP can modulate PKC ζ activity and/or be inactivated by small t, since activation of CAPP by ceramide could represent a possible mechanism of retrocontrol of PKC ζ signaling. In this context, Guy *et al.* (1995) have also proposed that TNF-α induces the inactivation of a PP2A-like phosphatase in fibroblasts. The fact that small t can substitute for TNF-α and activate PKC ζ raises the possibility that this phosphatase could be the enzyme inactivated by small t. Lastly, other positive or negative regulators of PKC ζ signaling include arachidonic acid, which inhibits basal and ceramide-stimulated PKC ζ activity (Müller *et al.*, 1995), phosphatidic acid, which binds to and activates PKC ζ (Limatola *et al.*, 1994), and the product of the *par-4* gene, which directly binds to and inhibits PKC ζ (Diaz-Meco *et al.*, 1996). Accumulation of ceramide (reviewed in Hannun, 1996) and expression of par-4 (Diaz-Meco *et al.*, 1996) induce cell cycle arrest and apoptosis. In contrast, PI 3-kinase promotes cell growth and survival (reviewed in Carpenter and Cantley, 1996). Thus, PKC ζ can be singled out as a central actor in a sophisticated signaling network that can transduce signals generating opposite biological effects. A stimulus-dependent modulation of PKC ζ signaling is probably achieved through a delicate balance involving lipid second messengers (ceramide, PIP3, arachidonic acid, phosphatidic acid), specific proteins (par-4), phosphatases (PP2A, etc.) and kinases (PI 3-kinase, etc.). Consequently, even minor changes in the spatial distribution, activity and/or intracellular levels of these different components may play a critical role in this fine regulation governed by protein–protein interaction.

In this study, we found that PKC ζ was required for the proliferative effects of small t in CV-1 and NIH 3T3 cells (Figure 2). Inhibition of PI 3-kinase nearly abolished small t-dependent cell growth (Figure 6). Together with the observation that small t-induced cell proliferation is mostly ERK2 dependent (Sontag *et al.*, 1993; Figure 2), these data suggest that the linear pathway involving PI 3-kinase/PKC ζ/MEK1/ERK2 primarily accounts for the ability of small t to promote cell growth. However, wortmannin affected small t-, but not serum-dependent cell growth (Figure 6A), indicating that alternate pathways are utilized following serum stimulation. It is well established that Raf-1 is a major Ras-responsive MEK kinase regulating the MAP kinase signal transduction cascade (reviewed in Marshall, 1995). It is noteworthy that, like PKC ζ (Cai *et al.*, 1997), small t is unable to activate Raf-1 (Sontag *et al.*, 1993). Based on these observations, we favor a model wherein two differentially regulated mitogenic pathways converge to MEK1 and ERK2 activation: one involves PI 3-kinase and PKC ζ, and is under the control of PP2A; the other one is mediated by direct activation of Raf-1 by Ras in a PP2A-independent fashion. Our data agree with the proposition that both Raf-1 and PKC ζ are located on separate branches of a mitogenic cascade (Bjorkoy *et al.*, 1995), and complement this study by demonstrating for the first time the existence of a differential modulation by PP2A of two cell growth-promoting pathways. It may prove difficult to investigate the contribution of PKC ζ in cell proliferation, because of the redundancy of these two pathways, and the fact that the Raf-1/MEK/ERK cascade is actively recruited following growth factor stimulation. Consistent with this notion, we found that kinase-deficient PKC ζ prevented small t-dependent growth (Figure 2), but had no apparent effect on serum-stimulated growth of quiescent CV-1 or NIH 3T3 cells (not shown). We thus believe that a significant role for PKC ζ in cell growth regulation can only be unveiled in serum-starved cells, under conditions where proliferation is triggered through a Raf-independent pathway. Indeed, the nature of PKC ζ involvement in mitogenesis and transformation remains a confusing and controversial issue. For instance, Gomez *et al.* (1995) have documented that PKC ζ controls interleukin-2-mediated T-cell proliferation. Berra *et al.* (1993) have suggested that overexpression of PKC ζ decreases the requirement for mitogens for cell proliferation in NIH 3T3 cells, but in other studies it did not affect the growth properties of NIH 3T3 cells stimulated with growth factors or transformed by Ras (Crespo *et al.*, 1995; Montaner *et al.*, 1995), and even reverted transformation by the v-*raf* oncogene (Kieser *et al.*, 1996). Lastly, overexpression of PKC ζ in prostate cancer cells inhibited metastasis (Powell *et al.*, 1996). The remarkable complexity of PKC ζ regulation underlines the possible difficulties in extrapolating a unique role for PKC ζ from overexpression studies performed in different cellular environments, like normal, transformed or tumoral cells, as illustrated above. In addition, most of these groups have examined the behavior of overexpressed PKC ζ in Ras-transformed cells, or in cells where mitogenesis occurs in response to activation of Raf-1 by growth factors. In contrast, we address the biological function of endogenous PKC ζ in a model where the growth of serum-starved cells is not stimulated by activation of the Ras/Raf/MEK/ERK cascade, but is entirely dependent on small t/PP2A. Using this distinctive approach, we demonstrate here that small t activates endogenous PKC ζ, and that this activation is responsible for a severe deregulation of the growth properties of CV-1 and NIH 3T3 cells, including reduced dependence on serum, lower doubling times and increased saturation density (Sontag *et al.*, 1993; Figure 2). It is striking that PKC ζ produces identical effects in NIH 3T3 cells in the

study by Berra *et al.* (1993). Although we cannot explain the reasons for certain discrepancies concerning the role of PKC ζ in NIH 3T3 cell growth (Berra *et al.*, 1993; Montaner *et al.*, 1995), our data support the hypothesis that PKC ζ participates in the control of mitogenic signaling.

TNF-α stimulation of PKC ζ previously has been connected to I-κB α degradation and NF-κB activation (Diaz-Meco *et al.*, 1993, 1994; Dominguez *et al.*, 1993; Lozano *et al.*, 1994). Incubation of human HeLa and T cells with low concentrations of okadaic acid induce similar effects (Sun *et al.*, 1995; Traenkner *et al.*, 1995). Instead of okadaic acid, which can inhibit several isoforms of PP2A and PP1 *in vivo*, our experiments relied on the utilization of wild-type or mutant small t proteins, which are capable or not of specifically inhibiting the PP2A ABαC heterotrimer *in vitro* and *in vivo* (Sontag *et al.*, 1993). Our results clearly establish that degradation of I-κB α (Figure 3A) and subsequent activation of NF-κB-driven gene expression (Figure 3B) occur in response to specific inhibition of PP2A by small t. However, they do not rule out the possibility that other phosphatases control NF-κB-mediated promoter gene activation. Our data support a role for PKC ζ (Figure 4) and PI 3-kinase (Figure 7) in the regulation of small t-induced NF-κB-dependent gene transcription. One simple mechanism by which small t and PKC ζ could activate NF-κB could be through direct activation of ERK2. However, we find this unlikely, because overexpression of dominant-negative ERK2 has no effect on NF-κB-dependent gene transactivation by either small t or TNF-α (Figure 4B and D). These findings agree with the previous work from J.Moscat's laboratory (Diaz-Meco *et al.*, 1994), but challenge the conflicting data recently reported by the same group (Berra *et al.*, 1995). Our results favor the hypothesis that two distinct kinases, a putative I-κB α kinase (Diaz-Meco *et al.*, 1994) and MEK1, become activated following inhibition of PP2A, and are involved in divergent pathways downstream of PKC ζ and PI 3-kinase.

The mechanisms by which small t enhances the transformation of resting cells are probably related to its ability to stimulate or repress the transcription of certain genes. In addition to AP-1 (Frost *et al.*, 1994) and CREB (Wheat *et al.*, 1994), we identify NF-κB as a new transcription factor targeted by small t. The physiological significance of this finding is multiple because NF-κB regulates the transcriptional activation of genes critical for various cellular processes (reviewed by Bauerle and Baltimore, 1996). First, PKC ζ-mediated NF-κB activation is responsible for persistent HIV-1 infection in monocytes (Folgueira *et al.*, 1996). Okadaic acid induces NF-κB-dependent HIV-1 LTR transcriptional up-regulation in Jurkat cells (Thévenin *et al.*, 1990). Using small t, we demonstrate that specific inhibition of PP2A is sufficient to activate NF-κB-dependent HIV-1 LTR transcription significantly by stimulating PKC ζ (Figure 3C). These data point to a novel key mechanism by which inhibition of PP2A could enhance HIV-1 transcriptional activation *in vivo*. Next, a role for NF-κB in cell growth control has been suggested (Baldwin *et al.*, 1991). Activation of NF-κB-dependent gene transcription could therefore account for some proliferative effects of small t. Remarkably, Yamaoka *et al.* (1996) have shown that the constitutive activation of NF-κB is essential for transformation of rat fibroblasts by the human T-cell leukemia virus I Tax protein, suggesting that small t-induced NF-κB activation could also be critical for cell transformation by SV40. Finally, cells often respond to the systemic stress induced by TNF-α by undergoing apoptosis or growth arrest. However, TNF-α also triggers a negative feed-back mechanism in which activation of NF-κB induces genes that are anti-apoptotic (Van Antwerp *et al.*, 1996). It has therefore been suggested that viruses inducing NF-κB could protect against apoptotic elimination of infected cells (Bauerle and Baltimore, 1996). Viruses could strategically evade a host's natural defense by interfering with the response of infected cells to pro-apoptotic extracellular signals, like TNF-α. Consistent with these hypotheses, SV40 small t can substitute for TNF-α and activate NF-κB in quiescent cells, as well as potentiate the effects of TNF-α on gene transcriptional activation. Induction of NF-κB-controlled genes by small t may therefore be extremely decisive for maximal replicative success of SV40 during infection of CV-1 cells. In the same context, it is riveting that cells transformed by polyomaviruses display an altered susceptibility to TNF-α, and that polyomavirus small t antigen, which interacts with PP2A *in vivo*, protects the transformed cells against TNF-α-induced cytotoxicity (Bergqvist *et al.*, 1997). Results obtained with SV40 small t uncover the possible general mechanism of this anti-apoptotic effect of polyomavirus small t.

In conclusion, we show here for the first time that inhibition of PP2A by SV40 small t in CV-1 and NIH 3T3 cells stimulates two divergent PI 3-kinase-dependent PKC ζ signaling pathways leading to NF-κB-dependent gene transcriptional activation, and activation of the mitogenic MAP kinase cascade. DNA tumor viruses, like SV40, only encode a limited number of proteins that are responsible for ensuring viral progeny. They have thus evolved to alter cellular behavior with maximum efficiency by targeting molecules critical for growth control of the infected cells. Accordingly, SV40 small t is capable of forcing quiescent cells to re-enter the S phase of the cell cycle and stimulate DNA replication in the permissive CV-1 cells, thus leading to an optimal intracellular environment for viral replication (Cicala *et al.*, 1994). Activation of the PKC ζ cascade by deregulation of PP2A following expression of small t in CV-1 cells is an attractive model to explain the mechanisms underlying the role of small t in SV40 transformation. Altogether, our findings support a critical role for PP2A/PKC ζ in fundamental cellular processes targeted during infection by SV40 and HIV-1.

Materials and methods

Reagents

Plasmids utilized in this study included: ConA-luc, κB-ConA-luc and HIV-1 LTR-luc luciferase reporter vectors (Bachelerie *et al.*, 1991), pCMV5 or Rc/CMV vectors for expression of small t proteins and ERK2-Y185F (Sontag *et al.*, 1993), pRcCMV-ζPKC^{mut} (Berra *et al.*, 1993) and SRα-Δp85 (Hara *et al.*, 1994). Other reagents included antibodies to PKC ζ (Santa Cruz Biotechnology), I-κB α and NF-κB (Dr R.Hay, St Andrews University, Scotland). Human TNF-α was donated by the MRC AIDS directed Programme Reagent Project.

Cell culture and transfection

Monkey kidney CV-1 and mouse NIH 3T3 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 5% Cosmic calf serum (Hyclone). Subconfluent cell cultures (100 mm dishes) were transiently transfected with the indicated plasmids using lipofectamine, according to the manufacturer's instructions (Gibco BRL). Transfections were carried out at a constant amount of DNA (15 μg/100 mm dish). For experiments involving luciferase reporter assays, transfections also included an additional 1 μg of the reporter plasmid pCMV-βgal as a monitor for transfection efficiency (Sontag *et al.*, 1993).

To obtain CV-1 cells stably expressing small t, 50% confluent cultures were transfected with RcCMV-small t, and clones of stable transfectants were isolated for their ability to grow in the presence of geneticin (Gibco BRL). Expression of small t was verified by immunoblotting and immunofluorescence microscopy using a monoclonal anti-small t antibody, as described previously (Sontag *et al.*, 1993). Corresponding control cells were obtained after selection of cells transfected with the empty vector RcCMV. Cells stably expressing RcCMV were found to behave like normal non-transfected cells (not shown).

Assay of PKC ζ activity

Cells (100 mm dishes) were serum-starved by incubation for 24 h in DMEM containing 0.1% serum, then left untreated or stimulated for 10 min with 10% serum or 10 ng/ml TNF-α. In some experiments, cells were pre-incubated for 1 h with 100 nM okadaic acid (Sigma) or for 5 h with either 10–500 nM wortmannin (Sigma) or 10–100 μM LY294002 (Calbiochem). PKC ζ activity was measured in immunoprecipitates by measuring the incorporation of ^{32}P into the synthetic peptide $MBP_{4–14}$ (Upstate Biotechnology Inc.), a specific PKC substrate (Yasuda *et al.*, 1990) or recombinant MEK-1 (Diaz-Meco *et al.*, 1994), under the conditions described previously (Yasuda *et al.*, 1990). Briefly, cell extracts were normalized for protein concentration and pre-cleared for 1 h at 4°C with 30 μl of protein A-coated Sepharose beads (Gibco BRL). Immunoprecipitation was performed at 4°C by incubating the fractions for 2 h with 4 μl of anti-PKC ζ, then for 1 h with 30 μl of the beads. Control experiments were performed by carrying out the immunoprecipitations with anti-PKC ζ antibody that had been pre-adsorbed with the corresponding PKC ζ peptide (1:2 ratio). After extensive washing, the immunoprecipitates were resuspended in 30 μl of a buffer containing 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 10 μM ATP and 0.5 μCi of [γ-^{32}P]ATP, and incubated for 15 min at 30°C with 25 μM $MBP_{4–14}$ or 1 μg of MEK-1, in the presence or absence of 10 μM of a PKC ζ inhibitor peptide (Upstate Biotechnology Inc.). Reaction mixtures were spotted onto P81 phosphocellulose paper and washed with 75 mM orthophosphoric acid. Incorporation of ^{32}P was determined by liquid scintillation.

Analysis of MEK phosphorylation in CV-1 cell lysates

Transfected cells (100 mm dishes) were serum-starved by incubation for 24 h in DMEM containing 0.1% serum. Total cell extracts were then prepared as described previously (Sontag *et al.*, 1993). Equivalent amounts of proteins (~40 μg) were resolved on SDS–12% polyacrylamide gels, immunoblotted and analysed for the phosphorylation status of MEK using the 'PhosphoPlus MEK1/2 (Ser217/221) Antibody' kit from New England Biolabs. Immunoblots were probed with an antibody that reacts with MEK independently of its phosphorylation state, and a phospho-specific MEK antibody, which recognizes active MEK only when phosphorylated at Ser217/221.

Preparation of nuclear extracts and Western blot analysis

At 48 h after transfection, cells (100 mm dishes) were washed once with phosphate-buffered saline (PBS) and incubated for 5 min in a buffer containing 0.25 M sucrose and 1 mM EDTA, pH 7.4. The buffer was removed, and cells were lysed for 15 min in 1 ml of buffer (0.25 M sucrose, 1 mM imidazole, pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), then Dounce homogenized. Nuclei were isolated by centrifugation (800 *g*, 10 min) through the sucrose cushion, washed twice and resuspended in the same buffer. Cytosolic fractions were obtained after recentrifugation of the supernatants for 10 min at 12 000 *g*. Equivalent amounts of proteins (~70 μg) from the cytosolic and nuclear fractions were resolved on SDS–14% polyacrylamide gels, and analyzed for the expression of either I-κB α, p110, small t or PKC ζ proteins by Western blotting. Immunoblots were developed using the ECL chemiluminescence detection method (Amersham Corp.).

Luciferase reporter gene activity assays

Cells were serum starved for 20 h in DMEM containing 0.1% fetal serum, incubated for 4 h in serum-free DMEM in the presence or absence of 5 ng/ml TNF-α, then harvested for luciferase assays. In some experiments, cells were pre-incubated for 3 h with 100 nM okadaic acid or for 5 h with 100 nM wortmannin or 75 μM LY294002. Luciferase activity was determined 48 h post-transfection by using the Luciferase Assay System kit from Promega. Total cell extracts (900 μl) were prepared according to the manufacturer's instructions. Luciferase activity was measured in duplicate aliquots (20 μl) of cell extracts by measuring for 10 s the light emission in an Opticomp luminometer. Correction for potential differences in transfection efficiency between plates within an experiment was performed by normalizing the luciferase activity to the β-galactosidase activity, which was determined in the same sample as described previously (Sontag *et al.*, 1993).

Cell proliferation assay

Cells were seeded 24 h post-transfection at the indicated density in DMEM supplemented with 10% serum. Cells were counted after 4 h to confirm accurate plating, at which time the medium was removed and replaced with DMEM containing either 0.5 or 10% serum. Cells were cultured for 0–72 h in the absence or presence of the indicated concentrations of wortmannin or LY294002, and harvested at the indicated time by trypsinization. Cell number was determined accurately by triplicate counting of aliquots from duplicate dishes using an automatic Coulter cell counter.

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