Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 306-311

PROTEIN KINASE C INCREASES THE ACTIVITY OF THE POLYOMA VIRUS MIDDLE T ANTIGEN-ASSOCIATED PHOSPHATIDYLINOSITOL KINASE

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Received June 2, 1988

Summary: Exposure of polyoma virus-transformed fibroblasts to the protein kinase C-stimulating phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) is known to increase the transforming potential of the virus's middle T antigen. Here it is shown that this TPA treatment also stimulates an 85 kDa phosphatidylinositol kinase associated with the middle T antigen. Since activation of this kinase is known to be necessary, although not by itself sufficient for the transformation of cells by polyoma virus, bursts of protein kinase C activity, triggered by TPA or various cellular receptors, might enhance the oncogenicity of polyoma virus by stimulating this middle T antigen-associated phosphatidylinositol kinase.

One of the early proteins of the oncogenic polyoma virus, middle T antigen (mT), is necessary for the transformation of established cell lines (1,2). In the cell, middle T antigen forms a complex with $pp60^{c-src}$ (3) and $p62^{c-yes}$ (4). This interaction results in an increase in the tyrosine protein kinase activity of $pp60^{c-src}$, partly because mT stabilizes a form of $pp60^{c-src}$ which is **not** phosphorylated at position 527, the major tyrosine phosphorylation site of $pp60^{c-src}$, which is thought to negatively regulate the enzyme's kinase activity (5-9). Extensive genetic and biochemical evidence has indicated that this increase in the kinase activity of the $pp60^{c-src}$ through mT binding is necessary, although not by itself sufficient for transformation by polyoma virus (9-11).

Triggering a burst of protein kinase C activity in polyoma virus-transformed fibroblasts with the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), stimulates the phosphorylation of mT in mT:$pp60^{c-src}$ complexes, immunoprecipitated from lysates of the treated cells by anti-mT antibodies (12-14). It also enhances the transforming ability of mT, as indicated by a dramaticaly increased ability of TPA-treated cells producing submaximal amounts of mT to proliferate while suspended in semi-solid medium and to form foci on confluent monolayers of normal cells (13).

The biochemical pathway by which protein kinase C thus modulates the oncogenicity of polyoma virus was investigated further. It was recently shown that cell transformation by polyoma virus results in phosphorylation of an 85 kDa phosphoprotein on tyrosine in vivo, as

0006-291X/88 $1.50

well as in vitro, on anti-phosphotyrosine or anti-mT immunoprecipitates (10, 15). This phosphorylation parallels an increase in phosphatidylinositol (PI)-kinase activity in mT-containing immunoprecipitates, as well as an increase in PI metabolism in cells expressing transformation-competent mT antigen molecules (11, 16). Therefore, it has been suggested that the 85 kDa PI-kinase phosphoprotein is an in vivo substrate of the mT:pp60^{c-src} kinase, which is activated by tyrosine phosphorylation (10, 15). In this communication we show for the first time that this protein kinase C-mediated activation of the mT:pp60^{c-src} complex which increases the transforming potential of mT, also increases the phosphorylation of the 85 kDa protein and its associated phosphatidylinositol kinase activity.

MATERIALS AND METHODS

Cell lines, culture techniques and mT analysis

Cell lines, culture techniques, stimulation of protein kinase C and mT analysis have been described previously (2, 12, 13). In all experiments shown, the gels had been treated with alkali prior to autoradiography. The NmT-1 line was obtained by transfecting mouse NIH3T3 cells with a plasmid containing the mT gene downstream of the steroid-inducible promotor of the Mouse Mammary Tumor Virus long terminal repeat (MMTV-LTR), just as described for the rat F111-derived lines in (2). Induction of mT was achieved by overnight incubation in the presence of 0.5×10^{-6} M dexamethasone (2).

Phosphatidylinositol (PI)-kinase assays

They were performed essentially as described by Whitman et al (19), with minor modifications. In brief, clarified 1% NP-40 cell extracts were precipitated with anti-polyoma ascites fluid. Immunoprecipitates were subsequently washed 3 times with 0.5% NP-40 in phosphate-buffered saline to remove all traces of contaminating type II PI kinase (19) and twice with 20mM Hepes, pH 7.2. Immune complexes were then resuspended in 40 µl of 20 mM Hepes, pH 7.2, 5 mM MnCl2, 0.2 mg/ml phosphatidylinositol (dispersed by sonication), and 10 µCi γ^{32}P ATP. Following incubation at 20° C for 15 minutes, the reactions were stopped with the addition of 100 µl 1M HCl; phospholipids were extracted with 200 µl Chloroform-MeOH (1:1) and resolved by chromatography on silica gel plates in a Chloroform-MeOH-4M NH4OH (9:7:2) mixture. Cold phosphatidylinositol-4-monophosphate served as marker.

Phosphatidylinositol and phosphatidylinositol-4-monophosphate were from Sigma (cat # P2517 and P9638 respectively).

Tyrosine-kinase experiments were always performed at the same time. Every experiment was run in triplicate.

The 1G2 monoclonal antibody producing hybridoma line was obtained from A. R. Frackelton and the antibody was prepared as described in (20).

RESULTS

TPA-mediated protein kinase C stimulation in py6 cells constitutively expressing the polyoma early region (17), increased the phosphorylation of tyrosine residues on both the 56 and 58 kDa forms of mT (12-14 and Fig. 1B, lane 2) and, on the average, tripled the PI kinase activity of mT:pp60^{c-src} complexes precipitated from py6 cell lysates with anti-mT antibody (Fig. 1A, lane 3). The PI kinase stimulated by protein kinase C, converted PI specificaly to phosphatidylinositol-3-monophosphate (18 and Fig. 1A). Treatment of py6 cells with a TPA analog, 4 αphorbol-12,13-didecanoate (4αPDD), which doesnot stimulate protein kinase C significantly, didnot increase mT phosphorylation or PI kinase activity in mT:pp60^{c-src} immunoprecipitates (Fig. 1A, lane 2; Fig. 1B, lane 1). The PI kinase activity in the mT:pp60^{c-src} complexes was also stimulated by raising the intracellular Ca^{2+} with the Ca^{2+} ionophore

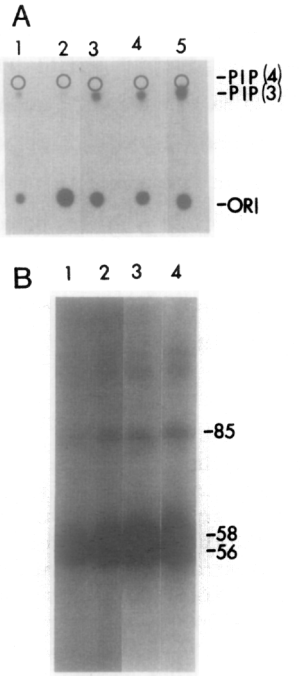

Figure 1: In vitro PI kinase activity (A) and 85 kDa protein phosphorylation (B) after protein kinase C stimulation in py6 cells. Cell extracts were incubated with anti-mT antibodies, while γ [^{32}P]-ATP, Mn^{2+} and sonicated PI were added to washed immunoprecipitates before thin layer chromatography (A) or gel electrophoresis (B, see Materials and Methods).
A: 1: No treatment; 2: 4αPDD; 3: TPA; 4: A23187; 5: TPA+A23187.
ori: origin of migration; PIP(4): position of the phosphatidylinositol-4-monophosphate marker; PIP(3): position of the phosphatidylinositol-3-monophosphate produced by the type I PI kinase.
B: 1: 4αPDD; 2: TPA; 3: A23187; 4: TPA+A23187.
Horizontal bars point to the 56 and 58 kDa forms of mT and the 85 kDa phosphoprotein.

A23187 (Fig. 1A, lane 4), and it was increased even further by treating the cells with both A23187 and TPA (Fig. 1A, lane 5).

The increase in PI kinase activity of the mT:pp60^{c-src} complexes from py6 cells treated with TPA, or with A23187 plus TPA, was accompanied by an increased phosphorylation of an 85 kDa protein on tyrosine (Fig. 1B, lanes 2-4). Since it is known that an 85 kDa type I PI kinase which phosphorylates PI to phosphatidylinositol-3-monophosphate binds to and is phosphorylated by the mT:pp60^{c-src} complexes in vitro, the 85 kDa protein in Fig. 1B, whose increased phosphorylation was associated with increased phosphatidylinositol-3-monophosphate production by the protein kinase C-activated mT:pp60^{c-src} complexes, is almost certainly the same enzyme.

As shown by Kaplan et al (16), the 85 kDa PI kinase can also be stimulated by phosphorylation on tyrosine residues by the activated PDGF receptor (15, 18). To verify whether PKC stimulation similarly affects the portion of the 85 kDa protein molecules which are normally associated with the PDGF receptor, we examined the phosphorylation of the 85 kDa protein present in immunoprecipitates prepared against the 1G2 monoclonal antibody to phosphotyrosine (20) from NIH 3T3 cells expressing the mT gene under control of the Dexamethasone-regulatable MMTV-LTR promotor (NmT-1 cells, see Materials and Methods). Indeed, the level of phosphorylation of the 85 kDa protein in complexes precipitated by the 1G2 anti-

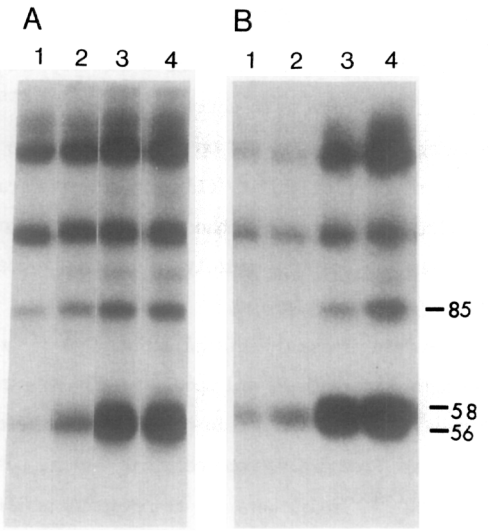

Figure 2. In vitro phosphorylation of proteins immunoprecipitated from the NmT-1 line of NIH3T3 cells with the 1G2 monoclonal antibody to phosphotyrosine. Washed immunoprecipitates were incubated with $\gamma[^{32}P]$ ATP and Mn^{2+} (see Materials and Methods).
A: Cells grown in 10% calf serum. Lanes 1-2: no mT induction. 3-4: mT induced. 1 and 3: no TPA treament. 2 and 4: TPA added.
B: Same as above, serum-starved cells.

phosphotyrosine antibody from lysates of uninduced NmT-1 cells containing only trace amounts of mT, depended on whether the cells had been proliferating in high serum (hence PDGF-rich)-medium or had been rendered proliferatively quiescent by incubation for two days in low serum (hence PDGF-poor)-medium before extraction. Thus, the 85 kDa protein was precipitated from cell lysates by 1G2 antibody when uninduced NmT-1 cells were proliferating in high serum-medium (Fig. 2A, lane 1). By contrast, there was very little or no tyrosine phosphorylation of the 85 kDa protein in lysates of quiescent, serum-deprived, uninduced NmT-1 cells (Fig. 2B, lane 1).

Treating uninduced NmT-1 cells proliferating in high serum medium with TPA, increased the level of 85 kDa protein phosphorylation only slightly (approximately 50%) in 1G2 anti-phosphotyrosine immunoprecipitates (Fig. 2A, lanes 1 and 2). TPA treatment also did not affect the very low levels of 85 kDa protein phosphorylation in 1G2 immunoprecipitates from quiescent, serum (thus PDGF)-deprived uninduced NmT-1 cells (Fig. 2B, lanes 1 and 2).

Middle T induction in proliferating NmT-1 cells caused a twofold increase in in vitro 85 kDa protein phosphorylation in 1G2 immunoprecipitates (Fig. 2A, lanes 1 and 3). TPA treatment of the proliferating and mT antigen expressing NmT-1 cells did not further increase the already high levels of 85 kDa protein phosphorylation in the 1G2 immunoprecipitates (Fig. 2A, lanes 3 and 4). In quiescent, serum-deprived NmT-1 cells however, mT induction caused the 85 kDa phosphoprotein to appear in 1G2 immunoprecipitates (Fig. 2B, lane 3), and treating these cells with TPA greatly increased 85 kDa protein phosphorylation (Fig. 2B, lanes 3 and 4).

Finally, it should be noted that similar results were obtained with the mT-1 cell line, which is a rat F111 derivative expressing mT under control of the same MMTV promotor controling mT expression in the NmT-1 mouse cells (2).

DISCUSSION

Triggering an intracellular burst of protein kinase C activity with diacylglycerols or TPA in polyoma virus transformed cells increases the activity of the $pp60^{c-src}$ protein kinase in the mT:$pp60^{c-src}$ complexes which phosphorylates mT on tyrosine residues in vitro and greatly increases the viral protein's transforming ability (12-14). This indicates that the oncogenicity of the polyoma virus could also be enhanced by signals from various cellular receptors which stimulate polyphosphoinositide hydrolysis and the release of protein kinase C-stimulating diacylglycerols.

The exact nature of the protein kinase C-mediated activation of the mT:$pp60^{c-src}$ complex is not known. In cells overexpressing both mT and $pp60^{c-src}$, TPA treatment increased their association and in vitro kinase activity, but not in proportion to the components of the complex (LR, unpublished results). Presumably, some cellular factor, possibly the kinase which phosphorylates the tyr527 of $pp60^{c-src}$ and lowers its transforming ability and kinase activity (22), inhibits to some extent the binding of mT to the carboxy- end of $pp60^{c-src}$ (T. M. Roberts, personnal communication). Whether protein kinase C increases the mT-$pp60^{c-src}$ association by partially somehow relieving this block, is not known at the moment.

One of the enzymes involved in polyphosphoinositide metabolism is an 85 kDa type I PI kinase which catalyses the phosphorylation of PI to phosphatidylinositol-3-monophosphate (10, 15). In non-transformed cells, this enzyme associates with PDGF receptors and is stimulated by the receptor's tyrosine protein kinase activity (15, 18, 19). According to the present observation, and in aggreement with Sturani et al (23), the PDGF receptor:85kDa PI kinase system is not affected by protein kinase C. However, this PI kinase also associates with the mT:$pp60^{c-src}$ complexes (15, 18, 19) which are known targets of protein kinase C (12-14). Consequently, the stimulation of the $pp60^{c-src}$ kinase activity in mT:$pp60^{c-src}$ complexes by a TPA-induced burst of PKC activity results in tyrosine phosphorylation and stimulation of the associated PI kinase. Although the physiological function of the phosphatidylinositol-3-monophosphate produced by this kinase is unknown, we can at least conclude that mT:$pp60^{c-src}$ complexes are directly involved in the control of polyphosphoinositide metabolism and therefore might modulate signals from various mitogen receptors. Indeed, the activation of PI kinase by the mT:$pp60^{c-src}$ complexes is necessary for cellular transformation by polyoma virus, and stimulation of this activity may be the reason why protein kinase C enhances mT's transforming ability (13).

ACKNOWLEDGEMENTS

We wish to thank Dave Kaplan for advice and help with the PI kinase assays and A.R. Frackelton for providing us with the MA1G2 hybridoma. J. B. is the recipient of a MRC of Canada Scholarship. The financial support from the National Cancer Institute of Canada through grant #446 to L.R. is greatfully acknowledged.

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