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Phosphatidylinositol turnover and transformation of cells by Abelson murine leukaemia virus

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The transforming protein of the Abelson murine leukaemia virus encodes a protein-tyrosine kinase. Previously, we have shown that in Abelson-transformed cells, the Abelson kinase regulates the phosphoserine content of ribosomal protein S6. Phorbol 12-myristate 13-acetate (TPA), which activates protein kinase C, induces the phosphorylation of S6 at the same five phosphopeptides as found in S6 isolated from Abelson-transformed cells. We have investigated three models whereby the Abelson kinase might regulate S6 phosphorylation via the activation of protein kinase C. First, the Abelson kinase could phosphorylate protein kinase C on tyrosine. However, we do not detect significant amounts of phosphotyrosine in protein kinase C *in vivo*. Second, it has been suggested that protein-tyrosine kinases might phosphorylate phosphatidylinositol. This could increase the intracellular levels of diacylglycerol and thereby activate protein kinase C. Our data strongly suggest that direct phosphorylation of phosphatidylinositol by the Abelson protein-tyrosine kinase has no physiological role. Third, an indirect activation of protein kinase C may occur via an increase in the rate of phosphoinositide breakdown. We have found that phosphoinositide breakdown appears to be constitutively activated in Abelson-transformed cells. The implications of these observations are discussed with regard to S6 phosphorylation and the mechanism of Abelson-induced transformation.

Key words: Abelson murine leukaemia virus/cell transformation/phosphatidylinositol/protein kinase C/protein-tyrosine kinase

Introduction

Nearly half of the known oncogenes (reviewed in Hunter and Cooper, 1985), as well as the membrane receptors for epidermal growth factor (Ushiro and Cohen, 1980), platelet-derived growth factor (Ek and Heldin, 1982; Ek *et al.*, 1982), insulin-like growth factor-1 (Rubin *et al.*, 1983) and insulin (Avruch *et al.*, 1982; Kasuga *et al.*, 1982), encode protein-tyrosine kinase activities (reviewed in Foulkes and Rich Rosner, 1985). These findings have suggested that the phosphorylation of proteins on tyrosine residues plays a critical role in the control of cell growth. However, physiologically important substrates for these enzymes have yet to be identified in any of these systems (Cooper and Hunter, 1983a; Foulkes and Rich Rosner, 1985).

All of the above protein kinases appear to be specific for tyrosine residues *in vitro*, but the addition of epidermal growth factor (Thomas *et al.*, 1982), platelet-derived growth factor (Nishimura and Deuel, 1983) or insulin (Kasuga *et al.*, 1982; Swergold *et al.*, 1982; Thomas *et al.*, 1982) to responsive cells also results in the increased phosphorylation of certain proteins on serine residues. Similarly, in cells transformed by Abelson murine leukaemia virus (A-MuLV) or Rous sarcoma virus (RSV), both of which encode protein-tyrosine kinases, an increase in protein-bound phosphoserine has been observed (Decker, 1981; Cooper and Hunter, 1983b; Maller *et al.*, 1985). Among these phosphoseryl-proteins, ribosomal protein S6 is of particular interest because its phosphorylation is correlated with growth-promoting stimuli in a wide variety of systems (Haselbacher *et al.*, 1979; Thomas *et al.*, 1982; Nishimura and Deuel, 1983).

Previously, we have shown that the phosphorylation of S6 in NIH 3T3 fibroblasts is dependent on the presence of serum but, after transformation of these cells by A-MuLV, S6 is highly phosphorylated on serine residues either in the absence or presence of serum. These results imply that the protein-tyrosine kinase encoded by A-MuLV can bypass the requirement for the growth factors in serum which normally regulate S6 phosphorylation (Maller *et al.*, 1985).

In the course of these experiments, we had observed that the phorbol ester phorbol 12-myristate 13-acetate (TPA), which is known to activate protein kinase C and induce S6 phosphorylation (LePeuch *et al.*, 1983; Nishizuka, 1984; Trevillyan *et al.*, 1984), induces the phosphorylation of the same five S6 phosphopeptides as found in Abelson-transformed cells (Maller *et al.*, 1985). Furthermore, protein kinase C can phosphorylate S6 directly *in vitro* (LePeuch *et al.*, 1983; Parker *et al.*, 1985). This suggested that the Abelson kinase might regulate S6 phosphorylation by activation of protein kinase C. We were very intrigued, therefore, by the reports that the protein-tyrosine kinases encoded by UR2 virus and RSV might also phosphorylate phosphatidylinositol, leading to the production of phosphatidylinositol 4,5-bisphosphate (Macara *et al.*, 1984; Sugimoto *et al.*, 1984).

It has been proposed that hydrolysis of phosphatidylinositol 4,5-bisphosphate, by phospholipase C, generates two potential second messengers (Berridge, 1984), namely diacylglycerol, which activates protein kinase C (Nishizuka, 1984), and inositol 1,4,5-trisphosphate, which appears to release calcium from intracellular sites and activate calmodulin-dependent pathways (Berridge and Irvine, 1984; Irvine *et al.*, 1984).

We speculated that the Abelson kinase might regulate the phosphorylation of S6 by phosphorylation of phosphatidylinositol, increasing the level of diacylglycerol and thereby activating protein kinase C. Given that multiple control mechanisms are likely to be involved in any pathway important in the transformation process, we have also examined the possibility that protein kinase C might be a direct substrate of the Abelson tyrosine kinase *in vivo*.

Here we present evidence which suggests that the direct phosphorylation of phosphatidylinositol by the Abelson protein-tyrosine kinase has no physiological role. Furthermore, we have failed to detect significant amounts of phosphotyrosine in protein kinase C in cells transformed by A-MuLV. We have found, however, that phosphatidylinositol turnover appears to be constitutively acti-

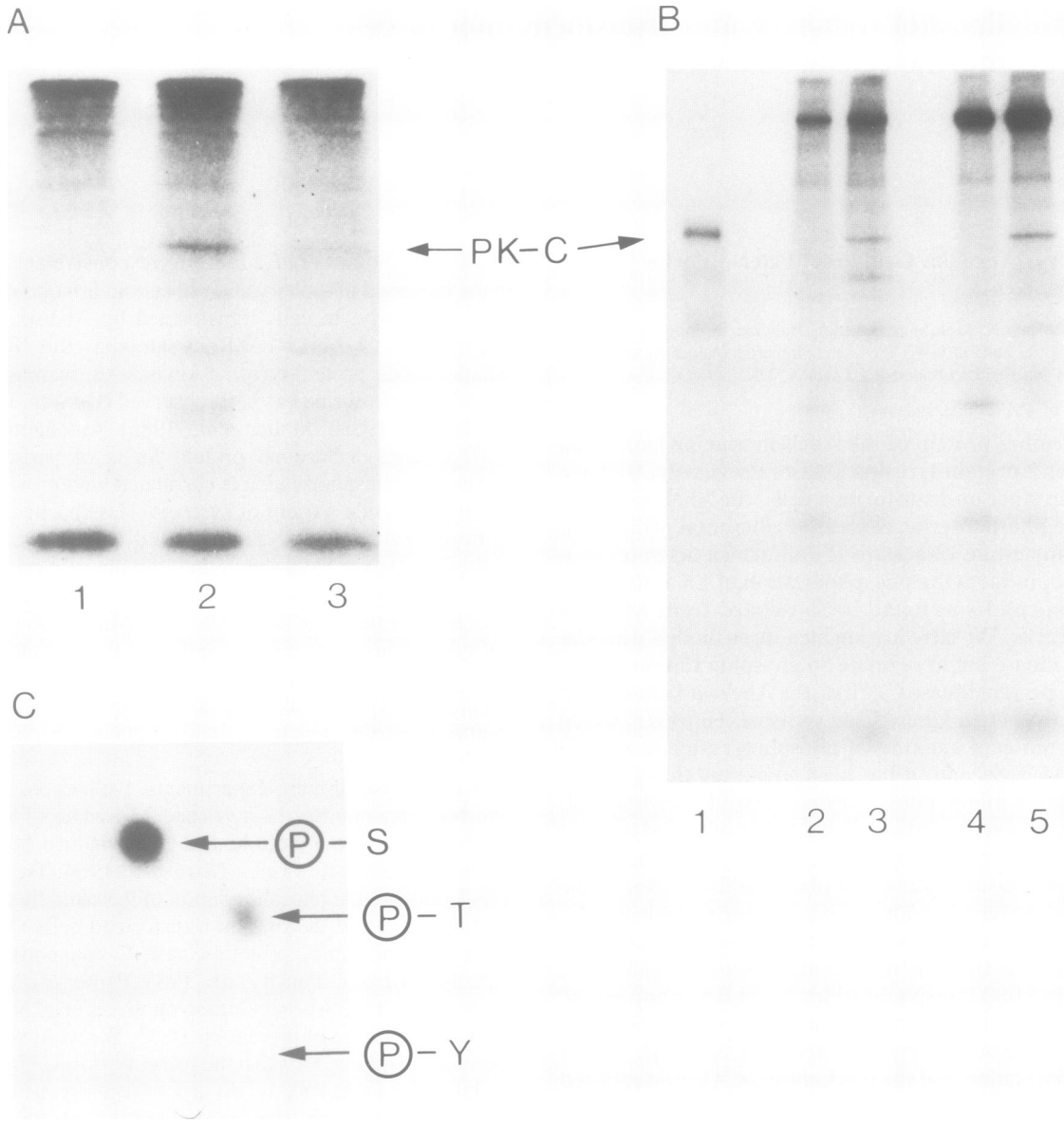

Fig. 1. Phosphorylation of protein kinase C *in vivo*. NIH 3T3 and ANN-1 cells were ^{32}P-labelled, protein kinase C immunoprecipitated and analysed by autoradiography following SDS gel electrophoresis as described in the text. **Panel A.** Immunoprecipitation of protein kinase C from NIH 3T3 cells with pre-immune serum **(lane 1)**, immune serum **(lane 2)** and immune serum in the presence of 2 μg purified protein kinase C **(lane 3). Panel B.** Immunoprecipitation of protein kinase C with either pre-immune serum **(lanes 2** and **4)** or immune serum **(lanes 3** and **5)**. Immunoprecipitates from NIH 3T3 cells **(lanes 2** and **3)** and ANN-1 cells **(lanes 4** and **5). Lane 1** shows purified protein kinase C autophosphorylated *in vitro*. The data were normalised to the total trichloroacetic acid precipitable counts incorporated into each cell type. **Panel C.** Phosphoamino acid analysis of protein kinase C immunoprecipitated from ANN-1 cells.

vated in Abelson-transformed cells. The implications of these observations are discussed.

Results

Our lysis buffer (see Materials and methods) has been designed to minimise post-homogenisation phosphorylation-dephosphorylation events. EDTA inhibits all known protein kinases. EDTA-fluoride inhibits phosphoseryl-phosphothreonyl protein phosphatases. SDS, deoxycholate, β-glycerophosphate and vanadate inhibit the dephosphorylation of phosphotyrosine (Foulkes and Rich Rosner, 1985). Figure 1 shows that the protein kinase C antibodies immunoprecipitate specifically one major phosphoprotein (mol. wt. 80 kd) from ^{32}P-labelled Abelson-transformed NIH 3T3 cells. This protein co-migrates on one-dimensional SDS gel electrophoresis with purified protein kinase C autophosphorylated *in vitro* (Figure 1B). The ability of purified protein kinase C to compete specifically the binding of this protein to the protein kinase C antibodies further substantiates the identity of this immunoprecipitated polypeptide as protein kinase C (Figure 1A). Immunoprecipitation of protein kinase C from both ^{32}P-labelled NIH 3T3 and Abelson-transformed NIH 3T3 cells (ANN-1) indicated that there was no significant change in the amount of ^{32}P-labelled protein kinase C between normal and Abelson-transformed cells (Figure 1B). Phosphoamino acid analysis of the immunoprecipitated protein kinase C revealed that phosphoserine and a smaller amount of phosphothreonine were the major phos-

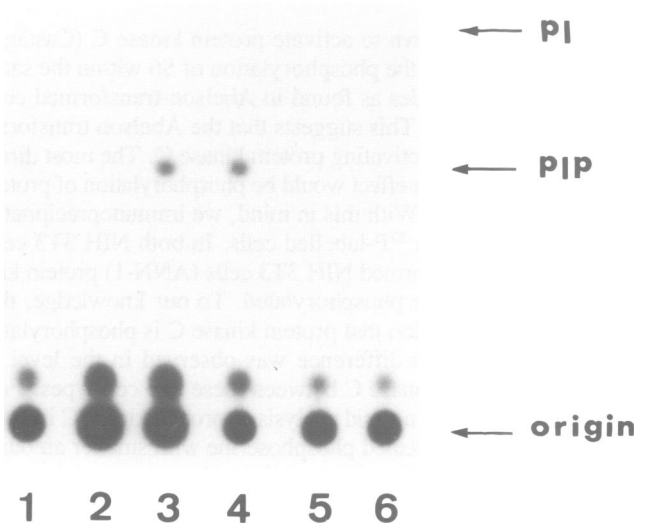

Fig. 2. *In vitro* phosphorylation of phosphatidylinositol by immunoprecipitates containing the Abelson protein-tyrosine kinase. The Abelson protein-tyrosine kinase was immunoprecipitated from ANN-1 cells, and incubated with phosphatidylinositol and [γ-^{32}P]ATP as described. The figure shows an autoradiograph of ^{32}P-labelled products separated by chromatography in buffer system A as detailed in the text. **Lanes 1** and **2**: immunoprecipitates from ANN-1 cells prepared with pre-immune serum; **lanes 3** and **4**: immunoprecipitates from ANN-1 cells prepared with anti-Abelson protein kinase serum; **lanes 5** and **6**: immunoprecipitates incubated with phosphatidylinositol and ATP and prepared in the absence of cell lysates.

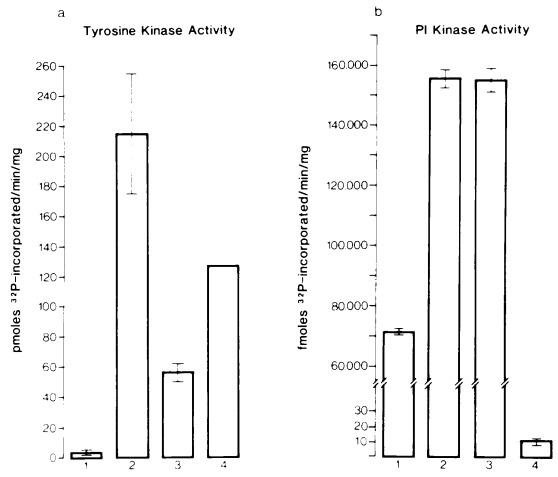

Fig.3. Panel A. Protein-tyrosine kinase activity of NIH 3T3 and ANN-1 cell lysates. Protein kinase activity in **lane 1**, NIH 3T3 extracts; **lane 2**, ANN-1 extracts before the immunoprecipitation of the Abelson kinase; **lane 3**, ANN-1 extracts after the immunoprecipitation of the Abelson kinase; **lane 4**, protein-tyrosine kinase activity of immunoprecipitates containing the Abelson tyrosine kinase. **Panel B.** Phosphatidylinositol kinase activity in NIH 3T3 and ANN-1 cell lysates. **Lane 1**, phosphatidylinositol kinase activity in NIH 3T3 extract; **lane 2**, phosphatidylinositol kinase activity in ANN-1 extracts before the immunoprecipitation of the Abelson kinase; **lane 3**, phosphatidylinositol kinase activity in ANN-1 extracts after immunoprecipitation of the Abelson kinase; **lane 4**, phosphatidylinositol kinase activity of immunoprecipitates containing the Abelson kinase. In both **panels A** and **B** the relative kinase activities of the various lysates and immunoprecipitates were standardised to the protein concentration of the extract. The bars show standard errors from three independent experiments.

phorylated amino acids in protein kinase C isolated from either Abelson-transformed cells (Figure 1C) or NIH 3T3 cells (data not shown). Protein kinase C, autophosphorylated in the presence of [γ-^{32}P]ATP shows a similar composition of phosphoamino acids (data not shown). Phosphoamino acid analysis of the corresponding gel region from the pre-immune lane revealed only very small traces of either phosphoserine or phosphothreonine.

Our method of assaying for phosphatidylinositol kinase activity is based on that described previously for the immunoprecipitated oncogene product of UR2, p68$^{v\text{-}ros}$ (Macara *et al.*, 1984). Incubation of the immunoprecipitated Abelson protein kinase with [γ-^{32}P]ATP and phosphatidylinositol resulted in the formation of a ^{32}P-labelled species that co-migrates on two t.l.c. systems with authentic phosphatidylinositol 4-monophosphate. Figure 2 shows the data for chromatography system A. The kinase activity observed in these immunoprecipitates was in the range 8 fmol/min/mg of lysate protein (Figure 2, lanes 3 and 4; Figure 3b, lane 4). The phosphorylation of phosphatidylinositol was found to proceed linearly over a period of at least 20 min at 30°C (data not shown). No phosphatidylinositol 4-monophosphate was detectable in assays lacking cell lysate (Figure 2, lanes 5 and 6) or in the absence of phosphatidylinositol (data not shown). When lysates from NIH 3T3 cells were immunoprecipitated with anti-Abelson antibodies, or when lysates from NIH 3T3 (data not shown) or ANN-1 cells (Figure 2, lanes 1 and 2) were immunoprecipitated with a goat pre-immune serum only very low levels of phosphatidylinositol kinase activity were detectable. The nature of the two ^{32}P-labelled spots migrating near the origin has not been determined. They appear to vary irreproducibly with different batches of ATP and from one experiment to the next, regardless of our ability to measure reproducibly the phosphatidylinositol kinase activity of the immunoprecipitates, e.g., see Figure 2, lanes 2–4. The extensive washing procedure used in the preparation of the immunoprecipitates (see Materials and methods) was found to be necessary to obtain a reproducible difference in the phosphatidylinositol kinase between the immune and pre-immune reactions.

Next we examined the levels of both the phosphatidylinositol kinase activity (Figure 3b) as well as the protein-tyrosine kinase activity (Figure 3a) in the cell lysates before and after removal of the Abelson protein kinase by immunoprecipitation. The protein-tyrosine kinase activity (determined using angiotensin II as a synthetic substrate) is ~50-fold higher in the extracts from Abelson-transformed cells (Figure 3a, lane 2) compared with the very low levels of activity measurable in NIH 3T3 cells (Figure 3a, lane 1). Immunoprecipitation of the Abelson tyrosine kinase removed >75% of the total angiotensin tyrosine kinase activity detectable in the ANN-1 extract (Figure 3a, lane 3). Furthermore, 80% of the immunoprecipitated protein kinase was found to retain its enzyme activity in the immune complex (Figure 3a, lane 4).

In contrast to the 50-fold increase in protein-tyrosine kinase activity, we observed only a 2.2-fold stimulation of the phosphatidylinositol kinase activity in the extracts from Abelson-transformed cells compared with normal NIH 3T3 cells (Figure 3b, lanes 1 and 2). Immunoprecipitation of >75% of the Abelson protein had no effect on the level of phosphatidylinositol kinase activity in the ANN-1 extracts (Figure 3b, lane 3). Furthermore, the phosphatidylinositol kinase activity of immunoprecipitates containing the Abelson protein represents only 1:15 000 of the total potential phosphatidylinositol kinase activity of the cell (Figure 3b, lanes 2 and 4).

We have also determined the rate of turnover of the inositol phosphates in normal and Abelson-transformed cells (Figure 4). The last stage of inositol phosphate breakdown, the dephosphorylation of inositol 1-phosphate to free inositol, can be blocked with

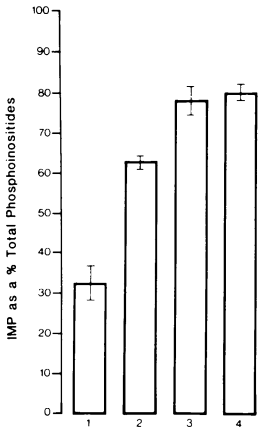

Fig. 4. *In vivo* labelling of inositol phosphates. ANN-1 cells and NIH 3T3 cells were labelled with [^{3}H]inositol, incubated with Li^+ for 30 min, followed by treatment for 10 min with or without serum, and inositol monophosphate isolated as described in the text. **Lane 1** (NIH 3T3 cells) and **lane 3** (ANN-1 cells) represent inositol monophosphate isolated from unstimulated cells grown for 8 h in the presence of 0.5% serum. **Lane 2** (NIH 3T3 cells) and **lane 4** (ANN-1 cells) were treated with serum (10% final) for 10 min. The data were normalised to the total counts incorporated and are expressed in terms of the percentage of total ^{3}H c.p.m. in the form of inositol monophosphate. The bars show standard errors from three independent experiments.

10 mM lithium chloride (Allison and Blisner, 1976). Thus, the percentage of the total inositol phosphates in the form of inositol monophosphate provides an estimate of the rate of phosphoinositide turnover (Allison *et al.*, 1976; Berridge *et al.*, 1982). After a 40 min incubation in the presence of Li^+, the percentage of inositol monophosphate in serum-starved ANN-1 cells was 2.5-fold higher than in serum-starved NIH 3T3 cells (Figure 4, lanes 1 and 3). Incubation of ANN-1 cells with 10% serum for 10 min after pre-incubation with Li^+ for 30 min had little effect on the percentage of the inositol phosphates in the form of inositol monphosphate (Figure 4, lanes 3 and 4). In contrast, incubation of NIH 3T3 cells with serum for 10 min led to a 2-fold increase in the percentage of the inositol phosphates in the form of inositol monophosphate (Figure 4, lanes 1 and 2).

Discussion

Transformation of cells by retroviruses results in the alteration of a multitude of cell parameters (Hanafusa, 1977). The discovery that a large number of retroviral oncogenes encode proteins with protein-tyrosine kinase activities suggested a common mechanism via which many of the oncogenes might exert changes on a large number of cell parameters. Unfortunately, physiologically important phosphotyrosine-containing substrates relevant to transformation have yet to be identified in any system (Cooper and Hunter, 1983a; reviewed in Foulkes and Rich Rosner, 1985).

Despite the fact that the Abelson protein kinase phosphorylates tyrosine residues exclusively (Foulkes *et al.*, 1985a), transformation of cells by A-MuLV also results in the increased phosphorylation of certain proteins on serine residues. One of these proteins is the ribosomal protein S6 (Maller *et al.*, 1985). The exact role of S6 remains to be defined but phosphorylated S6 may function to stimulate cell growth via the acquisition and preferential translation of growth-associated mRNAs (Duncan and McConkey, 1982; Glover, 1982; Thomas *et al.*, 1982). The most direct mechanism whereby Abelson tyrosine kinase could regulate the phosphorylation of S6 on serine would be the activation of a serine-specific S6 kinase following phosphorylation of the S6 kinase on tyrosine.

TPA, which is known to activate protein kinase C (Castagna *et al.*, 1982), induces the phosphorylation of S6 within the same five S6 phosphopeptides as found in Abelson-transformed cells (Maller *et al.*, 1985). This suggests that the Abelson transforming protein might be activating protein kinase C. The most direct mechanism for such an effect would be phosphorylation of protein kinase C on tyrosine. With this in mind, we immunoprecipitated protein kinase C from ^{32}P-labelled cells. In both NIH 3T3 cells and in Abelson-transformed NIH 3T3 cells (ANN-1) protein kinase C was found to be phosphorylated. To our knowledge, this is the first demonstration that protein kinase C is phosphorylated *in vivo*. However, no difference was observed in the level of ^{32}P-labelled protein kinase C between these two cell types. Furthermore, phosphoamino acid analysis of protein kinase C isolated from ANN-1 cells revealed phosphoserine with smaller amounts of phosphothreonine. On a very prolonged exposure of the autoradiograph, a trace amount of ^{32}P-phosphotyrosine was detectable in protein kinase C from ANN-1 cells but not in protein kinase C from NIH 3T3 cells (data not shown). This may represent either a minor contaminant present in the immunoprecipitated protein kinase C which co-migrates on one-dimensional SDS gel electrophoresis, or a trace phosphorylation of protein kinase C on tyrosine. As different protein-tyrosine kinases show marked differences in substrate specificity (Foulkes *et al.*, 1985b), we have also analysed protein kinase C from *src*-transformed NIH 3T3 cells and again only observe a trace level of phosphotyrosine (data not shown). Our lysis buffer appears to inactivate all protein-tyrosine phosphatase activities (Foulkes, 1984; J.G.Foulkes, unpublished observations) so it seems unlikely that phosphotyrosine would be lost during our isolation of protein kinase C. These data indicate that the Abelson transforming protein does not modulate the activity of protein kinase C by the direct phosphorylation of this enzyme on tyrosine residues. This suggested that we should search for alternative mechanisms whereby the Abelson transforming protein might activate protein kinase C in ANN-1 cells.

Recently, a large number of hormones have been shown to stimulate phosphoinositide turnover (Habernicht *et al.*, 1981; Creba *et al.*, 1983; Downes and Wusteman, 1983; Rebecchi and Gershengorn, 1983; Brown *et al.*, 1984; Nishizuka *et al.*, 1984) and it has been suggested that the physiologically important targets for certain oncogenes may be components of phospholipid metabolism (Berridge and Irvine, 1984; Macara *et al.*, 1984; Sugimoto *et al.*, 1984; Berridge *et al.*, 1985; Macara, 1985; Whitman *et al.*, 1985). In particular, data have been presented to suggest that protein-tyrosine kinases might phosphorylate phosphatidylinositol directly. It was possible that the Abelson transforming protein could stimulate protein kinase C indirectly via phosphatidylinositol phosphorylation. This perturbation might cause an increase in the level of diacylglycerol in Abelson-transformed cells, which would activate protein kinase C and in turn regulate S6 phosphorylation.

Immunoprecipitates containing the transforming protein of A-MuLV are associated with a phosphatidylinositol kinase activity *in vitro* (Figure 2), but the observed reaction rate is only 8 fmol ^{32}P-incorporated into phosphatidylinositol/min/mg of lysate protein. This is very similar to the rate of phosphatidylinositol phosphorylation reported by Macara *et al.* (1984) for the p68$^{v\text{-}ros}$ protein immunoprecipitated from UR2 virus-transformed cells. Similar phosphatidylinositol kinase activities have also been observed in immunoprecipitates of the polyoma middle T/c-*src* complex (Whitman *et al.*, 1985) as well as in purified preparations

of $pp60^{v\text{-}src}$, the transforming protein of RSV. $pp60^{v\text{-}src}$, purified from RSV-transformed cells, phosphorylates phosphatidylinositol with a V_m determined at 12 nmol/min/mg (Sugimoto *et al.*, 1984). In contrast, the Abelson protein, purified from *Escherichia coli* using an expression vector system, has a protein-tyrosine kinase specific activity of 3 μmol/min/mg and yet fails to phosphorylate phosphatidylinositol (Foulkes *et al.*, 1985a). Phosphatidylinositol kinases with such low specific activities may be of questionable physiological significance particularly when one recalls that growth factor-stimulated phosphoinositide breakdown is one of the most rapid effects detectable, occurring within seconds following the addition of an agonist (Berridge, 1983; Creba *et al.*, 1983; Downes and Wusteman, 1983; Rebecchi and Gershengorn, 1983).

In contrast to the 50-fold increase in protein-tyrosine kinase activity following transformation by A-MuLV, only a 2.2-fold stimulation of the phosphatidylinositol kinase activity in extracts from ANN-1 cells could be observed. Furthermore, immunoprecipitation of >75% of the Abelson protein had absolutely no effect on the phosphatidylinositol kinase activity in ANN-1 cells. Although immunoprecipitates containing the Abelson protein express the majority of the protein-tyrosine kinase activity, they express only 1:15 000 of the total potential phosphatidylinositol kinase activity (Figure 3). Based on this analysis we consider it extremely unlikely that direct phosphorylation of phosphatidylinositol by the Abelson protein-tyrosine kinase has any physiological significance.

The observed phosphatidylinositol activity of immunoprecipitates containing the Abelson kinase may either represent a trace phosphorylation of phosphatidylinositol by the Abelson protein or a cellular phosphatidylinositol kinase which is partially immunoprecipitated. Due to the normal affinity of enzymes for their substrates, kinases and their protein substrates are frequently found in association with one another, even in highly purified preparations. It is possible that a cellular phosphatidylinositol kinase is a substrate for the Abelson kinase and hence is partially immunoprecipitated by the anti-Abelson antibodies. In view of the fact that the Abelson kinase purified from *E. coli* fails to phosphorylate phosphatidylinositol this latter possibility seems particularly attractive, and could explain the increase in the total phosphatidylinositol kinase activity present in Abelson-transformed cells.

To estimate the rate of phosphoinositide turnover *in vivo*, cells were incubated for a short time in the presence of lithium chloride to block the conversion of inositol monophosphate to free inositol (Downes and Michell, 1981; Berridge, 1983; Berridge *et al.*, 1983). Any stimulation of the phosphoinositide pathways is then reflected by an increase in the amount of inositol monophosphate. As shown previously, differences in S6 phosphorylation between normal and transformed cells are only apparent in the absence of serum (Maller *et al.*, 1985). Under these conditions S6 is dephosphorylated in NIH 3T3 cells but remains phosphorylated in ANN-1 cells. If the Abelson kinase regulated S6 phosphorylation by the activation of phosphatidylinositol turnover and thereby protein kinase C, differences in phosphoinositide turnover between normal and transformed cells might only be apparent in the absence of serum. Our approach, therefore, was to measure the rate of phosphoinositide turnover in the absence and presence of serum. In the absence of serum inositol monophosphate levels were 2.5-fold higher in ANN-1 cells than in NIH 3T3 cells. Following the addition of serum for 10 min there was a rapid stimulation of inositol monophosphate levels in NIH 3T3 cells with no significant effect in Abelson-transformed cells.

These data indicate that the phosphoinositide pathway is constitutively activated following transformation by A-MuLV. These results are supported by previous investigations of phosphatidylinositol metabolism in both UR2 virus and RSV-transformed cell lines (Diringer and Friis, 1977; Macara *et al.*, 1984). This could lead to a sustained increase in the steady-state concentrations of both diacylglycerol, leading to the activation of protein kinase C, and inositol trisphosphate, leading to the release of intracellular calcium. Other phosphoinositide metabolites, e.g., arachidonic acid, might also act on systems potentially important in the transformation process. We are now determining the steady-state levels of each of the phosphoinositide metabolites in both NIH 3T3 cells and in Abelson-transformed cells. This may indicate which step(s) is activated by the Abelson kinase. The phosphotyrosine content of the enzymes regulating phosphoinositide metabolism in Abelson-transformed cells will then be examined. The relationship between protein kinase C activation and S6 phosphorylation also remains to be established as it is unclear whether protein kinase C is directly responsible for the phosphorylation of S6 observed in Abelson-transformed cells *in vivo*. Finally, this work suggests that a valuable approach in studying the mechanism of cell transformation by A-MuLV will be to identify the physiological substrates of both protein kinase C and the Ca^{2+}-calmodulin-dependent kinases and phosphatases, as these may represent key targets in the transformation process.

Materials and methods

Methods

Newborn calf serum was obtained from Imperial Laboratories. [γ-^{32}P]Adenosine 5′ triphosphate was obtained from New England Nuclear (2900 ci/mmol) or from Amersham (3000 Ci/mmol). Myo-[2-^{3}H]inositol was obtained from Amersham (19.1 Ci/mmol). Protein A-Sepharose CL-4B, Nonidet P-40 (NP-40), phospholipids, angiotensin II, bovine serum albumin, ATP and Dowex-1 X8-400 (chloride form) were obtained from Sigma. Silica gel 60 t.l.c. plates were obtained from Merck. All other chemicals used were of analytical grade or better. *Staphylococcus aureus*, Cowan strain, was grown and fixed as described previously (Kessler, 1975).

Cell cultures and preparation of cell lysates

NIH 3T3 and ANN-1 cells (Scher and Siegler, 1975) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum. Tissue culture plates containing ~10^7 cells were washed twice in ice-cold phosphate-buffered saline (PBS) and the cells scraped into 1.0 ml of lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 50 mM NaF, 30 mM Na pyrophosphate, 100 μM Na vanadate, 1% Triton X-100, 1% NP-40, 20 mM β-mercaptoethanol and 2 mM EDTA) at 4°C. After 15 min on ice the lysates were centrifuged at 13 000 *g* for 10 min at 4°C. Cleared cell lysates were prepared by three 15 min incubations at 4°C with 5 μl of goat pre-immune serum and 50 μl of a 25% formaldehyde-fixed *S. aureus* cell suspension. After each incubation the bound material was removed by centrifugation at 13 000 *g* for 1 min. These cleared cell lysates were used for immunoprecipitation and for assaying protein-tyrosine kinase and phosphatidylinositol kinase activities in cell lysates. Protein concentrations were determined as described previously (Bradford, 1976).

Immunoprecipitation of the Abelson transforming protein

100 μl aliquots of the cleared cell lysates were incubated with 5 μl of the indicated antiserum for 2 h on ice. 20 μl of Protein A-Sepharose CL-4B (Sigma) (a 50% v/v slurry in lysis buffer) were added and mixed for 45 min at 4°C. The adsorbed immune complexes were then spun down in a microfuge and the supernatant removed. The Protein A-Sepharose pellet was washed once in 1 M NaCl, 20 mM Tris, 0.1% NP-40, once in 0.5 M LiCl, 0.1 M Hepes (pH 7.4), twice in cell lysis buffer and finally in 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA. The resulting pellets were assayed for either protein-tyrosine kinase or phosphatidylinositol kinase activity.

Determination of protein-tyrosine kinase activity in cell extracts and in immunoprecipitates

Protein tyrosine kinase activity was measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into the octapeptide angiotensin II. Assays were carried out as described previously (Foulkes *et al.*, 1985a) except that the reactions were terminated by the addition of 5% trichloroacetic acid (instead of phosphoric acid) and protein removed by centrifugation.

Determination of phosphatidylinositol kinase activity

Phosphorylation of phosphatidylinositol was performed with either immunoprecipitates from cleared cell lysates or with 2 μl of a 1/200 dilution of cleared cell lysates, both before and after immunoprecipitation of the Abelson protein-tyrosine kinase. 2 mg/ml phosphatidylinositol in 1% NP-40, 200 mM Hepes (pH 7.4) was pre-incubated at 4°C with the immune pellet for 20 min at a final concentration of 0.5 mg/ml phosphatidylinositol, 50 mM Hepes. After pre-incubation the phosphorylation reaction was started by addition of 20 μCi [γ-^{32}P]ATP and $MgCl_2$ to final concentrations of 50 μM ATP, 10 mM $MgCl_2$ in a final volume of 50 μl. The reaction mixture was incubated for 10 min at 30°C. The reaction was then terminated and the lipids extracted as described previously (Whitman *et al.*, 1985). The organic phase was redissolved in 30 μl of chloroform/methanol (4:1) and spotted onto a silica gel 60 t.l.c. plate (Merck) impregnated with 1% potassium oxalate. Phosphorylated products were separated using either system A, chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8), or system B, chloroform/methanol/ammonia/water (45:30:3:5), and visualised by autoradiography. Authentic lipids were run as standards on each plate and were visualised by exposure to iodine vapour.

Determination of inositol phosphate turnover in vivo

Confluent 10-cm dishes of cells were grown in 0.5% serum, inositol-free DMEM for 8 h. 5 μCi myo-[2-^{3}H]inositol/ml was added and the incubation continued for 5 h at which time LiCl was added to a final concentration of 10 mM. After 30 min the cells were treated with or without serum as specified in the figure legends and incubated for a final 10 min. Cells were washed once in PBS and then scraped in 0.72 ml of 0.03 N HCl. 2.7 ml of chloroform/methanol (1:2) was added and the mixture incubated on ice for 15 min. Finally 0.9 ml chloroform and 0.9 ml water were added and the resultant two phases separated by centrifugation at 2000 r.p.m. for 10 min.

The water-soluble inositol phosphates present in the upper phase were fractionated using a 1-ml Dowex-1 (formate form) column as described previously (Berridge, 1983). After passage of the inositol phosphate fraction through the Dowex column, the column was washed through with 15 ml of distilled water to remove free inositol, which fails to bind to the column. The inositol phosphates were eluted sequentially by using 5 mM disodium tetraborate/60 mM sodium formate (for glycerophosphoinositol), 0.1 M formic acid/0.2 mM ammonium formate (for inositol monophosphates), 0.1 M formic acid/0.4 M ammonium formate (for inositol bisphosphates) and 0.1 M formic acid/1 M ammonium formate (for inositol trisphosphates). In routine assays 15-ml fractions were collected, evaporated to dryness and their radioactivities determined by scintillation counting.

Immunoprecipitation and phosphoamino acid analysis of protein kinase C

Protein kinase C antibodies were prepared from rabbits inoculated with purified protein kinase C isolated from human spleen and purified as described for the bovine brain preparation (Parker *et al.*, 1984).

2 × 10^6 NIH 3T3 or ANN-1 cells were labelled for 3 h with 2 mCi ^{32}Pi in 5 ml of phosphate-free DMEM containing 10% dialysed newborn calf serum. The cells were then washed once with PBS and lysed in RIPA buffer [20 mM Na phosphate (pH 7.2), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 100 μM Na vanadate, 80 mM Na β-glycerophosphate, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS]. After two pre-precipitation steps with 10 μl normal rabbit serum and 50 μl 10% formaldehyde-fixed *S. aureus*, the lysates were treated for 2 – 12 h with 10 μl of antiserum prepared against purified protein kinase C. In the competition experiment, 2 μg purified protein kinase C were added to lysates during the immune precipitation. Immune complexes, bound to *S. aureus* bacteria, were washed four times with RIPA buffer, resuspended in SDS gel sample buffer and treated at 80°C for 5 min. SDS-polyacrylamide gel electrophoresis in 10% slab gels was performed as described (Laemmli, 1970) and the dried gels subjected to autoradiography. Protein kinase C was eluted from gel slices by digestion with proteinase K, hydrolysed for 2 h at 105°C with 6 N HCl, and two-dimensional phosphoamino acid analysis performed as described (Hunter and Sefton, 1980).

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