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# Common Elements in Growth Factor Stimulation and Oncogenic Transformation: 85 kd Phosphoprotein and Phosphatidylinositol Kinase Activity

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## Summary

**The phosphorylation of proteins on tyrosine in vivo and in vitro was examined in 3T3 cells stimulated by platelet-derived growth factor (PDGF) and transformed by polyoma middle T antigen (MTAg) by using an antibody directed against phosphotyrosine (P-tyr). Two common events were observed upon PDGF stimulation or MTAg transformation of cells: the appearance in the immunoprecipitates of an 85 kd phosphoprotein, and increased phosphatidylinositol (PI) kinase activity. In PDGF-stimulated cells, the 85 kd phosphoprotein and PI kinase activity appeared rapidly, within 1 min of growth factor addition. The PI kinase activity and 85 kd phosphorylation were also increased in anti-P-tyr immunoprecipitates from cells transformed by v-*fms* and v-*sis*, but not by SV40 T antigen. The presence of the tyrosine-phosphorylated 85 kd protein correlated with PI kinase activity during several purification steps. These results suggest that the 85 kd phosphoprotein, a putative PI kinase, is a substrate for both the PDGF receptor and MTAg/pp60$^{c\text{-}src}$ tyrosine kinase activities.**

## Introduction

An important feature of transformed cells is their partial or complete autonomy from growth factor requirements that limit the proliferation of their nontransformed counterparts. The growth factor independence of transformed cells has been postulated to be due to a constitutive expression of one or more of the signaling elements along the mitogenic pathway (Heldin and Westermark, 1984). Clues as to how oncogenes may regulate growth stimulatory pathways can be obtained by examining common structural and functional elements between the protein products of oncogenes and the transducers of growth factor signals. One of these common elements is protein-tyrosine kinase activity. Several growth factor receptors, including those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and colony stimulating factor 1 (CSF-1), are tyrosine kinases (Bishop, 1985). In each case, binding of ligand is known to stimulate the tyrosine kinase activity of the receptor. A number of oncogenes, including v-*src*, v-*abl*, v-*fms*, v-*fes*/*fps*, and polyoma virus middle T antigen (MTAg), encode proteins that are associated with plasma membranes and that possess intrinsic or associated tyrosine kinase activity (Hunter and Cooper, 1985). Phosphorylation of proteins on tyrosine residues has been offered as an explanation for the pleiotropic responses to growth factor and oncogenes (Hunter and Cooper, 1985; Bishop, 1985). Genetic analysis of the v-*src* and MTAg oncogenes suggests that tyrosine kinase activity is essential to the ability of these oncogenes to transform cells (Schaffhausen, 1982; Hunter and Cooper, 1985). In addition, cell growth stimulation by PDGF and cell transformation by v-*src* are both accompanied by increases in the level of tyrosine-phosphorylated proteins in cells (Cooper et al., 1982; Frackelton et al., 1984; Ek and Heldin, 1984; Hunter and Cooper, 1985; Morla and Wang, 1986). Some of the targets of tyrosine kinases have been identified (Hunter and Cooper, 1985). However, genetic analysis has shown that changes in the phosphorylation of the substrates identified to date are insufficient to account for the physiological changes accompanying mitogenesis and transformation (Hunter and Cooper, 1985).

One mechanism whereby growth factor receptors and oncogenes may exert their effects is through stimulation of cellular phosphatidylinositol (PI) turnover. Increased turnover of PI and its phosphorylated derivatives PIP and $PIP_2$ have been implicated in cellular responses to a wide variety of stimuli, including growth factors such as PDGF (Habenicht et al., 1981; Berridge et al., 1984, 1985; Whitman et al., 1987). Altered phosphorylation and turnover of PI has also been demonstrated in cells transformed by DNA and RNA tumor viruses (Koch and Diringer, 1973; Sugimoto et al., 1984; Macara et al., 1984; Fry et al., 1985; Kaplan et al., 1986; Jackowski et al., 1986; Kato et al., 1987). The hydrolysis of $PIP_2$ by phospholipase C produces two potent second messenger molecules, diacylglycerol (DG) and inositol trisphosphate ($IP_3$). $IP_3$ elevates cytosolic calcium by stimulating release from intracellular calcium stores (Berridge et al., 1984). DG directly activates the C-kinase, which is the major cellular receptor for the tumor-promoting phorbol esters (Nishizuka, 1984). The actions of DG and $IP_3$ have diverse effects on cell physiology, implicating these second messengers as possible effector molecules in growth factor stimulation and oncogenesis.

We have been investigating common elements in PDGF-mediated mitogenesis and transformation by polyoma middle T antigen. A number of parallels exist between the response of cells to PDGF and to MTAg. PDGF and MTAg stimulate the tyrosine kinase activity of pp60$^{c\text{-}src}$, the cellular homolog of the oncoprotein encoded by the v-*src* oncogene (Bolen et al., 1984; Ralston and Bishop, 1985).

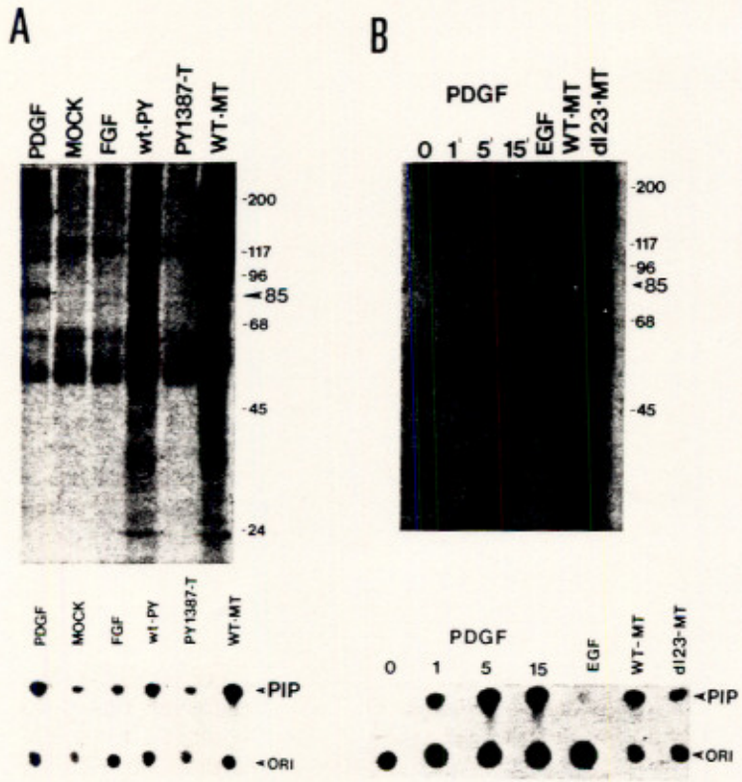


Figure 1. 85 kd Phosphorylation and PI Kinase Activities Assayed in Anti-P-tyr Immunoprecipitates from PDGF-Stimulated and MTAg-Expressing Cells

Anti-P-tyr immunoprecipitates were prepared from growth factor–treated or MTAg-expressing cells and assayed for tyrosine and PI kinase activities in vitro. (A) 85 kd phosphoprotein and PI kinase activities from quiescent BALB/3T3 cells treated for 15 min with 10 U/ml PDGF or 10 U/ml (4 ng/ml) FGF, infected with wild-type polyoma virus (wt-Py) or the transformation-defective polyoma virus PY 1387-T, or from NIH 3T3 cells transformed by MTAg (WT-MT). (B) Time course of the appearance of 85 kd phosphoprotein and PI kinase activity in PDGF-stimulated quiescent BALB/3T3 cells as assayed in the anti-P-tyr immunoprecipitates in vitro. Cells were treated for 0, 1, 5, or 15 min with 10 U/ml PDGF or for 15 min with 10 U/ml EGF at 37°C. Cells expressing wild-type MTAg (WT-MT) or the dl 23 mutant MTAg (MT-dl 23) were also assayed. Proteins were analyzed by 10% SDS–PAGE, and PI kinase reactions by TLC. PIP, phosphatidylinositol phosphate. ORI, origin.

While the mechanism and functional significance of PDGF activation of pp60$^{c\text{-}src}$ are not known, genetic and biochemical analysis suggests that the activation of pp60$^{c\text{-}src}$ kinase activity by complex formation with MTAg accounts for both the associated tyrosine kinase activity and a significant portion of the mechanism of action of MTAg (Courtneidge and Smith, 1984; Bolen et al., 1984). A second similarity is that PDGF and MTAg induce the tyrosine phosphorylation of a number of cellular proteins, including pp60$^{c\text{-}src}$ (Cooper et al., 1982; Ek and Heldin, 1984; Courtneidge and Smith, 1984; Ralston and Bishop, 1985; Cartwright et al., 1986; Yonemoto et al., 1987). Both PDGF and MTAg induce cellular PI turnover and $IP_3$ production in mouse 3T3 cells (Berridge et al., 1985; Kaplan et al., 1986). MTAg stimulation of $IP_3$ generation may be due in part to the regulation by MTAg of a cellular PI kinase. The appearance of this PI kinase in anti-MTAg immunoprecipitates prepared from MTAg-expressing cells correlates with the ability of MTAg to transform (Whitman et al., 1985; Kaplan et al., 1986). In addition, MTAg and PDGF stimulate the transcription of c-*myc* and c-*fos* (Kelly et al., 1983; Kruijer et al., 1984; Zullo et al., 1987). Finally, MTAg-transformed 3T3 cells will grow in platelet-poor plasma (Cherington et al., 1986), implying that MTAg alleviates the requirement of PDGF for fibroblast growth.

In this study, we investigated the phosphorylation of proteins on tyrosine in PDGF-treated and MTAg-transformed 3T3 cells by using anti-phosphotyrosine (anti-P-tyr) antiserum (White et al., 1985). Both MTAg and PDGF stimulated the appearance of a similar or identical 85 kd phosphoprotein in the anti-P-tyr immunoprecipitates. The presence of the 85 kd phosphoprotein coincided with increased PI kinase activity in the immunoprecipitates. These results suggest that one of the consequences of MTAg activation of pp60$^{c\text{-}src}$ or PDGF activation of its receptor's kinase activity may be the phosphorylation of an 85 kd protein, a putative PI kinase.

## Results

### An 85 kd Protein and Phosphatidylinositol Are Phosphorylated in Anti-P-tyr Immunoprecipitates from PDGF-Treated and MTAg-Expressing Cells

We examined the tyrosine and PI kinase activities in anti-P-tyr immunoprecipitates generated from cells expressing MTAg or treated with PDGF. Anti-P-tyr immunoprecipitates were prepared from quiescent BALB/3T3 cells (clone A31) treated for 15 min with PDGF (10 U/ml) or fibroblast growth factor (FGF) (10 U/ml), the 3T3 cells infected with transformation-competent (wt-Py) or transformation-defective (PY 1387-T) polyoma virus, and from MTAg-transformed NIH 3T3 cells (WT-MT). These immunoprecipitates were incubated with $MnCl_2$ and [$\gamma$-$^{32}$P]ATP, and the $^{32}$P-labeled proteins were resolved on SDS–PAGE (Figure 1A, top panel). An 85 kd phosphoprotein was immunoprecipitated from PDGF-stimulated cells and from cells expressing transformation-competent MTAg (WT-MT and wt-Py), but not from FGF-treated cells or cells expressing transformation-defective MTAg (PY 1387-T). The appearance of the phosphorylated 85 kd protein correlated with an increase

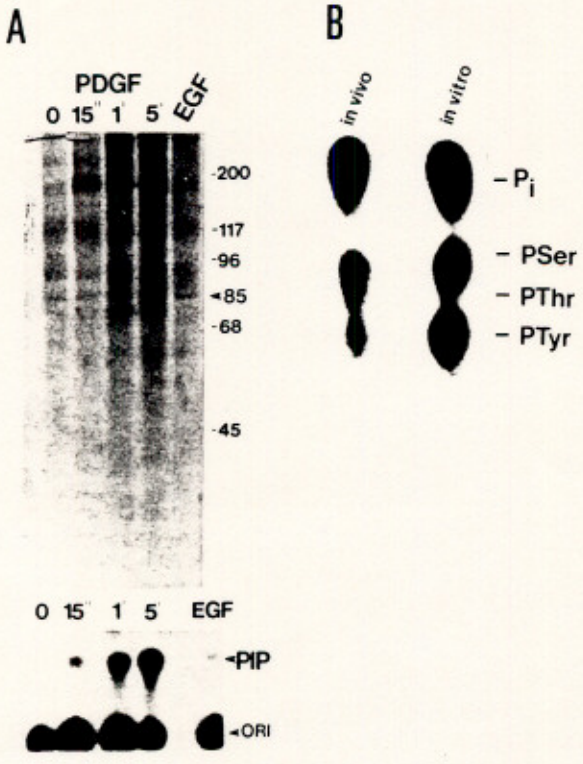


Figure 2. 85 kd Phosphoprotein in Anti-P-tyr Immunoprecipitates from PDGF-Stimulated Cells Labeled In Vivo

Quiescent BALB/3T3 cells were treated for 15 sec, 1 min, or 5 min with 10 U/ml PDGF or for 15 min with 15 ng/ml EGF at 37°C. To analyze the proteins phosphorylated in vivo, cells were labeled with [$^{32}$P]orthophosphate for 3 hr prior to growth factor treatment. Anti-P-tyr immunoprecipitates were prepared from cell lysates and analyzed directly on 10% SDS–PAGE or assayed for PI kinase activities in vitro. PI kinase reactions were analyzed by TLC. PIP, phosphatidylinositol phosphate. ORI, origin. (B) Phosphoamino acid analysis of the 85 kd proteins phosphorylated in vivo or in vitro in anti-P-tyr immunoprecipitates from PDGF-stimulated cells. Phosphoamino acid analysis was performed as in Experimental Procedures. PSer, phosphoserine. PThr, phosphothreonine. PTyr, phosphotyrosine.

in anti-P-tyr-associated PI kinase activity (Figure 1A, bottom panel). The anti-P-tyr immunoprecipitates from PDGF-stimulated or MTAg-transformed cells showed 10- to 20-fold increases in PI kinase activity as compared to mock-treated cells or cells expressing the PY 1387-T mutant MTAg. In addition, anti-P-tyr immunoprecipitates from cells expressing the transformation-defective dl23 MTAg mutant had reduced amounts of 85 kd protein phosphorylation and PI kinase activity compared to immunoprecipitates from wild-type MTAg-transformed cells (Figure 1B).

Since PDGF is known to induce PI turnover and tyrosine-specific phosphorylations within minutes (Berridge et al., 1985; Cooper et al., 1982), we determined the time course of the appearance of 85 kd protein phosphorylation and PI kinase activity in anti-P-tyr immunoprecipitates from PDGF-stimulated cells. Quiescent BALB/3T3 cells were treated with PDGF for various amounts of time, cell lysates were prepared, and the P-tyr-containing proteins were immunoprecipitated from the lysates with anti-P-tyr antiserum. The immunoprecipitates were labeled in vitro with [$\gamma$-$^{32}$P]ATP or, alternately, were assayed for PI kinase activity. Both phosphorylated 85 kd protein and enhanced PI kinase activity appeared rapidly, within 1 min of PDGF addition (Figure 1B). The increases in phosphorylation and PI kinase activity (50-fold) reached a maximal level 5 min after PDGF treatment and was not evident after 12 hr (Figure 1B and data not shown). The dose of PDGF producing half-maximal increases in both 85 kd phosphorylation and PI kinase activity in the immunoprecipitates was between 3 and 10 U/ml (6–20 ng of protein/ml). This is comparable to the dose required for half-maximal stimulation of DNA synthesis (data not shown). Treatment of quiescent BALB/3T3 cells for 15 min with EGF (15 ng/ml), insulin (250 μg/ml), tumor-derived growth factor (B-TGF) (10 ng/ml), or poly(I):poly(C) (50 μg/ml) did not result in increases in 85 kd protein phosphorylation or PI kinase activity as assayed in vitro (Figure 1B and data not shown).

### An 85 kd Phosphoprotein Is Observed in Anti-P-tyr Immunoprecipitates from Cells Labeled In Vivo and Treated with PDGF

To determine whether similar changes in the appearance of the 85 kd protein occur in vivo, quiescent BALB/3T3 cells were labeled in vivo with [$^{32}$P]orthophosphate prior to PDGF treatment and anti-P-tyr immunoprecipitation. Several phosphoproteins of 75–90 kd appeared in these immunoprecipitates in parallel with the appearance of PI kinase activity within 1 min of PDGF treatment (Figure 2A). One of these species comigrated with the 85 kd protein labeled in vitro (arrow in Figure 2A, top panel). The time course of the appearance of phosphate in the 85 kd protein in vivo (Figure 2A, top panel) was the same as that of the 85 kd protein labeled in vitro (Figure 1B, top panel). The 180 kd phosphoprotein observed in the anti-P-tyr immunoprecipitates from PDGF-stimulated cells (Figure 2A, top panel) is probably the PDGF receptor, based on the reported molecular weight of the receptor (Daniel et al., 1985) and the rapid phosphorylation of the protein following PDGF treatment of cells.

Phosphoamino acid analysis was performed on the 85 kd proteins phosphorylated in vivo or in vitro in the anti-P-tyr immunoprecipitates from PDGF-stimulated cells. The 85 kd protein was phosphorylated in vivo on serine and tyrosine residues at a ratio of approximately four to one (Figure 2B). The protein labeled in vitro was phosphorylated on tyrosine and serine residues at approximately equivalent amounts (Figure 2B). In the same experiments, the PDGF receptor was labeled in vitro almost exclusively on tyrosine residues.

The identity of the 85 kd proteins labeled in vitro with $^{32}$P in the anti-P-tyr immunoprecipitates from PDGF-stimulated cells and MTAg-transformed cells was determined by peptide mapping with S. aureus V8 protease. As shown in Figure 3 (lanes 1 and 2), the same series of six peptides ranging from 20 kd to 7.5 kd in size was derived from the 85 kd phosphoprotein in each case. Comparable results were obtained using chymotrypsin or cyanogen bromide (data not shown).

The relationship between the 85 kd proteins labeled in

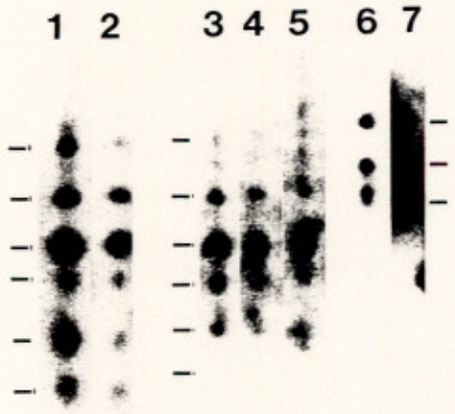


Figure 3. Peptide Mapping of the 85 kd Protein Phosphorylated In Vivo and In Vitro from PDGF-Treated and MTAg-Transformed Cells

Peptide mapping of the 85 kd proteins phosphorylated in vivo and in vitro in anti-P-tyr immunoprecipitates from PDGF-treated cells and MTAg-transformed cells. 85 kd proteins were digested with S. aureus V8 protease (lanes 1–5) or with chymotrypsin (lanes 6 and 7), and the peptides were resolved by SDS–PAGE. Lane 1, 85 kd protein labeled in vitro from MTAg-transformed cells. Lane 2, 85 kd protein labeled in vitro from PDGF-stimulated cells. Lane 3, 85 kd phosphoprotein phosphorylated in vitro from PDGF-treated cells. Lane 4, 85 kd protein phosphorylated in vivo from PDGF-treated cells. Lane 5, 85 kd phosphoprotein phosphorylated in vivo in anti-MTAg immunoprecipitates from MTAg-transformed cells. Lane 6, 85 kd protein phosphorylated in vitro in anti-P-tyr immunoprecipitates from PDGF-treated cells. Lane 7, 85 kd protein phosphorylated in vivo from PDGF-treated cells. [$^{32}$P]orthophosphate labeling, immunoprecipitation, and labeling in vitro were performed as described in Experimental Procedures. S. aureus V8 protease digestions were carried out at 40 μg/ml and the peptides were resolved by 12.5% SDS–PAGE. Chymotrypsin digestions were at 75 μg/ml and the peptides were resolved by 15% SDS–PAGE. The exposure times for the in vivo samples were 7 days with an intensifier screen. Exposure times for the in vitro samples were 2 days without an intensifier screen.

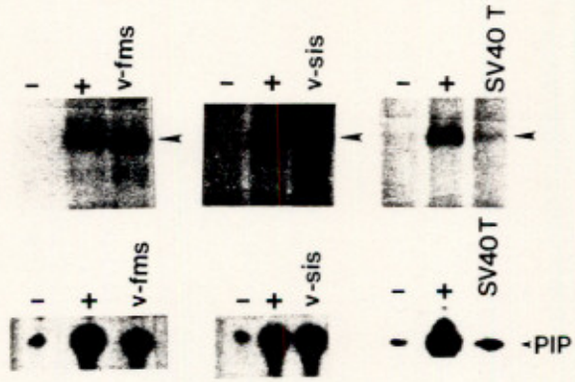


Figure 4. 85 kd Phosphorylation and PI Kinase Activities Assayed In Vitro in Anti-P-tyr Immunoprecipitates from Cells Transformed by v-*fms*, v-*sis*, and SV40 T Antigen

Anti-P-tyr immunoprecipitates were prepared from 3T3 cells transformed by the v-*fms*, v-*sis*, and SV40 T antigen oncogenes and assayed for tyrosine and PI kinase activities in vitro. The kinase activities in the transformed cell immunoprecipitates were compared in each case to the kinase activities in anti-P-tyr immunoprecipitates from PDGF-stimulated (+) or unstimulated (−) cells. Proteins were analyzed by 7.5% SDS–PAGE; PI kinase reactions, by TLC. The exposure times were the same for all samples. PIP, phosphatidylinositol phosphate.

vitro with [γ-$^{32}$P]ATP and in vivo with [$^{32}$P]orthophosphate was similarly addressed. Figure 3 (lanes 3 and 4) shows that the 85 kd protein labeled in vivo from PDGF-stimulated cells gave rise to the same major peptides as those seen after labeling in vitro; chymotryptic digestion also produced similar patterns for the in vivo– and in vitro–labeled 85 kd proteins (Figure 3, lanes 7 and 6). The proteins of 75 kd and 90 kd observed in anti-P-tyr immunoprecipitates from PDGF-treated cells labeled in vivo (Figure 2, top panel) shared no common peptides with the 85 kd phosphoprotein (data not shown). We next examined the 85 kd phosphoproteins from MTAg-transformed cells. An 85 kd phosphoprotein was detected in anti-P-tyr and anti-MTAg immunoprecipitates prepared from MTAg-transformed cells labeled in vivo with [$^{32}$P]orthophosphate. The 85 kd protein phosphorylated in vivo from MTAg-transformed cells shared a similar set of peptides with the 85 kd species labeled in vivo in anti-P-tyr immunoprecipitates from PDGF-treated cells (Figure 3, lanes 4 and 5).

### 85 kd Phosphoprotein and PI Kinase Activity in Other Transformed Cells

The structural and functional similarities between the v-*sis* oncogene and the PDGF B-chain (Doolittle et al., 1983; Waterfield et al., 1983) suggest that the phosphorylation of the 85 kd protein and PI kinase activity may be constitutively elevated in v-*sis*-transformed cells. We also examined v-*fms*-transformed cells since c-*fms*, the cellular homolog of the v-*fms* oncogene, has striking structural homology to the PDGF receptor (Yarden et al., 1986). Enhanced PI turnover has also been observed in v-*fms*-transformed cells (Jackowski et al., 1986). Cell lysates from the transformed cells were prepared, normalized for protein content, and immunoprecipitated with anti-P-tyr antiserum. The immunoprecipitates were analyzed for protein and PI kinase activities in vitro. As shown in Figure 4, immunoprecipitates from v-*sis*- and v-*fms*-transformed 3T3 cells contained increased levels of PI kinase activity and of phosphorylated 85 kd protein as compared to quiescent nontransformed 3T3 cells (−). Immunoprecipitates prepared from an SV40-transformed 3T3 cell line had little PI kinase activity or 85 kd phosphoprotein. Peptide mapping analysis indicated that the 85 kd proteins phosphorylated in the immunoprecipitates from v-*sis*- and v-*fms*-transformed cells were qualitatively identical to the 85 kd protein induced by PDGF treatment of quiescent 3T3 cells (data not shown).

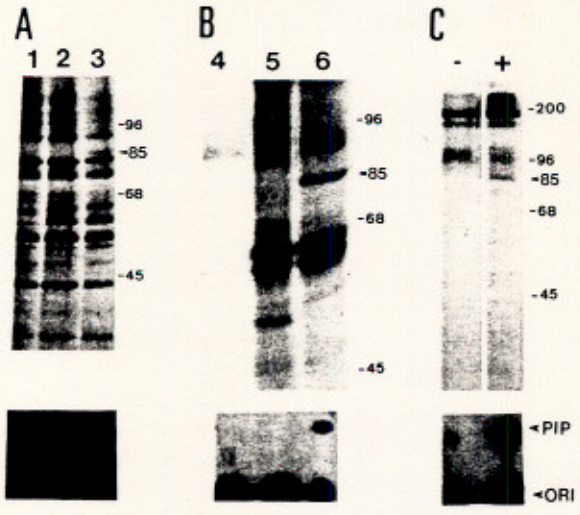


Figure 5. Correlation of the 85 kd Phosphoprotein with PI Kinase Activity through Several Purification Steps

(A) Elution of [$^{35}$S]methionine-labeled 85 kd protein and PI kinase activity from MTAg immunoprecipitates. Cell lysates from NIH 3T3 cells (lane 1) or NIH 3T3 cells expressing dl 23 mutant MTAg (lane 2) or wild-type MTAg (lane 3) were immunoprecipitated with anti-MTAg antiserum. The phosphoproteins were eluted as described in Experimental Procedures. Half of the sample was analyzed by 10% SDS–PAGE, and half was assayed for PI kinase activity. PI kinase reactions were extracted and analyzed by TLC. (B) Elution and anti-P-tyr reimmunoprecipitation of 85 kd phosphoprotein and PI kinase activity. Eluates were prepared from anti-MTAg immunoprecipitates as in (A). Prior to elution the immunoprecipitates were incubated with 20 μM [γ-$^{32}$P]ATP for 5 min. The eluates were reimmunoprecipitated with anti-P-tyr antiserum and analyzed by SDS–PAGE or for PI kinase activity. Lane 4, NIH 3T3 cells. Lane 5, NIH 3T3 cells expressing dl 23 mutant MTAg. Lane 6, wild-type MTAg. (C) 85 kd phosphoprotein and PI kinase activity obtained from PDGF-treated (+) and untreated (−) 3T3 cells using wheat germ lectin–agarose and anti-P-tyr chromatography. Cell lysates were incubated with wheat germ lectin–agarose and the precipitated proteins were phosphorylated in vitro with [γ-$^{32}$P]ATP. The proteins were eluted with N-acetylglucosamine and the eluate was immunoprecipitated with anti-P-tyr antiserum. The immunoprecipitates were divided and analyzed by SDS–PAGE or assayed for PI kinase activity. PIP, phosphatidylinositol phosphate. ORI, origin.

### The Presence of the 85 kd Protein Correlates with PI Kinase Activity through Several Purification Steps

We further examined the observed correlation of the PI kinase activity and 85 kd phosphoprotein using affinity chromatography. We have previously shown that anti-MTAg immunoprecipitates prepared from cells expressing MTAg contain an activity that phosphorylates PI (Whitman et al., 1985; Kaplan et al., 1986). Furthermore, we have been able to purify partially the PI kinase activity away from most of the MTAg/pp60$^{c\text{-}src}$ complex and other cellular proteins in the immunoprecipitates by extraction with a mixture of cholate, salt, and phospholipid (data not shown). MTAg-transformed cells labeled with [$^{35}$S]methionine were lysed and immunoprecipitations were performed with an anti-polyoma T antigen antiserum. As a control, parallel experiments were performed using the transformation-defective, PI kinase–defective mutant MTAg (dl 23), which is fully competent in activating pp60$^{c\text{-}src}$ as measured by enolase phosphorylation in vitro (Kaplan et al., 1986). The PI kinase activity was eluted from the immunoprecipitates, and the eluates were assayed for PI kinase activity and for labeled protein. Of the many species eluted in this procedure, only one band, at 85 kd, was observed to coelute with the PI kinase activity present in eluates from WT-MTAg (Figure 5A, lane 3), but not 3T3 (lane 1) or dl 23 cells (lane 2). To avoid the high background of [$^{35}$S]methionine-labeled bands, which could potentially obscure the PI kinase, we turned to a different labeling protocol. The immunoprecipitates were incubated with [γ-$^{32}$P]ATP to catalyze the phosphorylation of proteins by the MTAg/pp60$^{c\text{-}src}$ complex. The PI kinase activity was then eluted from the immunoprecipitates and the eluate was reimmunoprecipitated with anti-P-tyr antiserum. The anti-P-tyr immunoprecipitates were analyzed for PI kinase activity and labeled protein. Approximately half of the PI kinase activity in the eluate was precipitated by the anti-P-tyr antiserum, and the presence of the PI kinase activity correlated with a single labeled protein of 85 kd (which was also precipitated in a 50% yield) (Figure 5B, lane 6). Both the PI kinase activity and the 85 kd protein were absent in the anti-P-tyr immunoprecipitates from control cells (Figure 5B, lane 4) and from dl 23 MTAg-expressing cells (lane 5).

We also determined whether the PI kinase activity and the 85 kd phosphoprotein copurified during affinity chromatography of lysates prepared from PDGF-treated cells. We have previously shown that a PI kinase activity specifically associates with wheat germ lectin–agarose after addition of PDGF to quiescent 3T3 cells (Whitman et al., 1987). Lysates from PDGF-treated or untreated 3T3 cells were incubated with wheat germ lectin–agarose. The proteins bound to the lectin-agarose were phosphorylated with [γ-$^{32}$P]ATP and were then eluted from the reagent with N-acetyl glucosamine and octylglucoside. The eluate was immunoprecipitated with anti-P-tyr antiserum and the immunoprecipitates were assayed for PI kinase activity (Figure 5C, bottom panel) or analyzed directly by SDS–PAGE (Figure 5C, top panel). An 85 kd phosphoprotein and PI kinase activity are present in the anti-P-tyr immunoprecipitates from PDGF-treated (+), but not untreated (−), 3T3 cells. The PI kinase activity and the 85 kd phosphoprotein were nearly quantitatively immunoprecipitated with the anti-P-tyr antiserum. We estimate that 1%–2% of the cellular protein present in the wheat germ lectin precipitates was immunoprecipitated by the anti-P-tyr antiserum. Thus we achieved a 20- to 100-fold enrichment in PI kinase activity and in 85 kd phosphoprotein.

### Discussion

The substrates of oncogene and growth factor receptor tyrosine kinases are of interest because they are likely to be involved in the control of proliferation of normal and transformed cells. Using anti-P-tyr antiserum, we have examined the tyrosine phosphorylation of proteins in two systems where tyrosine kinases are activated, PDGF-stimulated 3T3 cells and cells transformed by polyoma MTAg. We have observed an 85 kd protein in anti-P-tyr immunoprecipitates that is phosphorylated in vivo and in vitro from cells treated for 1 min with PDGF. The rapid ap-

pearance of the 85 kd phosphoprotein in the immunoprecipitates following PDGF treatment suggests that this protein is a substrate of the PDGF receptor. The presence of the 85 kd phosphoprotein is increased in anti-P-tyr or anti-MTAg immunoprecipitates from cells containing transformation-competent MTAg, but is reduced or absent in immunoprecipitates from cells containing transformation-defective mutants of MTAg. Immunoprecipitates from cells transformed with an oncogene with structural similarities to the PDGF receptor, v-*fms* (Yarden et al., 1986), and with an oncogene that may transform cells by activating PDGF growth–stimulatory pathways, v-*sis* (Heldin and Westermark, 1984), also contained increased amounts of 85 kd phosphoprotein as assayed in vitro. Since the 85 kd protein and the PI kinase activity coimmunoprecipitate with the MTAg/pp60$^{c\text{-}src}$ and PDGF receptor tyrosine kinase activities, we do not know if the anti-P-tyr antiserum recognizes the 85 kd protein or the PI kinase activity directly. If the 85 kd protein were precipitated indirectly, this would explain the apparent paradox that arises when a protein immunoprecipitated by anti-P-tyr antiserum is phosphorylated on tyrosine residues in immune complex kinase assays. Alternatively, the 85 kd protein, like pp60$^{c\text{-}src}$, may have multiple sites of tyrosine phosphorylation, with different sites phosphorylated in vivo and in vitro. We do know that like pp60$^{c\text{-}src}$, the 85 kd protein is phosphorylated on tyrosine residues in vivo.

Proteins in the molecular weight range of the 85 kd protein have been previously observed in immunoprecipitates from polyoma virus–transformed cells and in growth factor–stimulated cells. A number of investigators have reported a protein of approximately 74 kd phosphorylated on tyrosine in response to PDGF addition to cells. Frackelton et al. (1984) have determined that this protein is cytosolic, with a pI of 4.2. The 85 kd protein reported here has a pI of approximately 7 (our unpublished data) and requires detergent for solubilization. An 81 kd protein (Ezrin) is phosphorylated on tyrosine in A431 cells stimulated by EGF (Gould et al., 1986); however, the 81 kd protein, when immunoprecipitated from cells using anti-Ezrin antibodies (obtained from A. Bretscher), migrates differently from the 85 kd protein observed here. These antibodies also fail to immunoprecipitate PI kinase activity. Other investigators have observed that a protein of 81 kd is phosphorylated in vitro on tyrosine residues in anti-MTAg immunoprecipitates from polyoma virus–transformed cells (Dilworth, 1982; S. Courtneidge, personal communication).

While a number of phosphotyrosine-containing proteins have been identified in polyoma virus–transformed and PDGF-stimulated cells, connecting the alterations in phosphorylation with known events of biological importance has been problematic. The coprecipitation of the 85 kd protein and the PI kinase activity from two different sources (PDGF-treated and MTAg-transformed cells) suggests such a connection. Both PDGF and MTAg stimulate striking increases in PI kinase activity in anti-P-tyr immunoprecipitates. In the case of MTAg, the increases in PI kinase activity and 85 kd protein phosphorylation are only observed in anti-P-tyr immunoprecipitates from cells expressing transformation-competent MTAg. In the case of PDGF, increased PI kinase activity and 85 kd phosphoprotein appear with the same time course and dose response. These data are consistent with the idea that the 85 kd protein is the PI kinase or one of its subunits. Assuming this is correct, we can estimate a specific activity of our most purified preparation of 85 kd protein at 300 nmol/min per mg (see Experimental Procedures). This value is five to ten times higher than the specific activity reported for the 45 kd PI kinase purified from bovine brain myelin (Saltiel et al., 1987). Although the 85 kd protein is the only band we observe to correlate with PI kinase activity in both MTAg/pp60$^{c\text{-}src}$ and PDGF receptor preparations, we cannot rule out the possibility that another protein corresponds to the PI kinase but is not detectable by our labeling protocols. Indeed, there are a number of less simple but perfectly plausible explanations for the identity and function of the 85 kd protein. For instance, our data are consistent with the possibility that the 85 kd protein is itself a tyrosine kinase, rather than a PI kinase. The 85 kd protein could in turn activate the PI kinase. To evaluate further the significance of the observed copurification of the 85 kd protein with the PI kinase activity, we are attempting to raise monoclonal antibodies to gel-purified 85 kd protein.

How may the changes in 85 kd phosphorylation and PI kinase activity that we observe in vitro be related to the changes in PI metabolism known to occur in PDGF-treated and MTAg-transformed cells? The initial step in PI turnover activation is thought to be the breakdown of $PIP_2$ by phospholipase C (Berridge et al., 1984). The observation that PIP, $PIP_2$, and $IP_3$ levels are elevated concomitantly in PDGF-stimulated (Berridge et al., 1984) and MTAg-expressing (Kaplan et al., 1986) cells suggests that PI and PIP kinases are activated in addition to phospholipase C. The changes in PI kinase activity that we observe in immunoprecipitates from PDGF-stimulated cells appear within 1 min of PDGF treatment. In contrast, MacDonald et al. (1987) reported that total cellular PI kinase activity increases in response to PDGF, but only after several hours of treatment. These investigators, however, did not distinguish between the different classes of PI kinase activity present in cells (Harwood and Hawthorne, 1969; Whitman et al., 1987). We have found that normal and transformed 3T3 cells have multiple PI kinase activities, one of which is capable of associating with MTAg/pp60$^{c\text{-}src}$ and other tyrosine kinases (Type I PI kinase) (Whitman et al., 1987). This activity is differentiated from other cellular PI kinases by its resistance to inhibition by adenosine and its sensitivity to inhibition by nonionic detergents. Since many investigators measure PI kinase activities in the presence of nonionic detergents, the PI kinase activity's association with tyrosine kinases may be obscured. While cells transformed by MTAg share some of the morphological and biochemical characteristics of cells treated with PDGF, PDGF-stimulated cells do not display the tumorigenic properties of MTAg-expressing cells (Westermark et al., 1983; Stiles, 1983; Ralston and Bishop, 1985; Kaplan et al., 1986). Thus the activation of PI turnover would be only one of a number of cellular processes that must occur for complete induction of malignancy.

At present, any detailed model concerning control of PI

kinase activity is speculative. Purification experiments (Figure 5) demonstrate that the 85 kd phosphoprotein copurifies with activated tyrosine kinases. In polyoma-transformed cells, the PI kinase associates with the MTAg/pp60$^{c\text{-}src}$ tyrosine kinase complex. In cells stimulated by PDGF, the PI kinase activity coimmunoprecipitates with the PDGF receptor and not with pp60$^{c\text{-}src}$, suggesting association with the receptor. While we detect increases in PI kinase activity in anti-pp60$^{c\text{-}src}$ immunoprecipitates from MTAg-transformed cells as compared to nontransformed cells, we do not observe increases in PI kinase activity in anti-pp60$^{c\text{-}src}$ immunoprecipitates from PDGF-stimulated cells (our unpublished data). This result suggests that the increases in PI kinase activity observed in the anti-P-tyr immunoprecipitates following PDGF treatment of cells occur in a manner independent of pp60$^{c\text{-}src}$. A model proposing regulation of PI kinase through direct phosphorylation by tyrosine kinases is consistent with these data. However, a number of other models could also be considered. For instance, since the 85 kd protein is phosphorylated on serine residues in vivo and in vitro, an indirect action of tyrosine kinases on serine kinases could occur. The relationship between the PI kinase activities observed in vitro and in vivo, and the nature of the links between tyrosine kinases and PI kinases, await the purification and characterization of the various PI kinases and the determination of their roles in cell growth and physiology.

## Experimental Procedures

### Cell Culture

BALB/3T3 clone A31 (from C. D. Scher, Children's Hospital, Philadelphia, PA), MTAg-transformed NIH 3T3 cells (Cherington et al., 1986), NIH 3T3 cells expressing dl 23 MTAg (from B. Morgan, Dana-Farber Cancer Institute), v-fms-transformed NIH 3T3 cells (from D. Morrison, Dana-Farber Cancer Institute), v-*sis*-transformed BALB/3T3 cells (from M. Armalin, H. Armalin, and C. D. Stiles, Dana-Farber Cancer Institute), SV40 T antigen–transformed NIH 3T3 cells (from M. Brown and D. Livingston, Dana-Farber Cancer Institute), and v-*src*-transformed NIH 3T3 cells (Piwnica-Worms et al., 1987) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn bovine serum. Experiments with BALB/3T3 cells were initiated on density-arrested monolayers as described previously (Cochran et al., 1983). Growth factors were added directly to the medium, and incubation continued at 37°C as stated in the figure legends. Transformed cell lines were not fed with fresh medium for at least 48 hr prior to use.

Infections with the wild-type polyoma virus NG59RA (Feunteun et al., 1976) and the polyoma virus mutant PY1387-T (Carmichael et al., 1982) were performed as described by Whitman et al. (1985).

### Mitogens

PDGF and EGF (receptor grade) were from Collaborative Research Inc. (Waltham, MA). Partially purified PDGF was provided by C. D. Stiles (Dana-Farber Cancer Institute). Acidic FGF (0.4 ng of protein per unit) was from P. A. D'Amore (Children's Hospital Medical Center, Boston, MA). PDGF and FGF were used at a concentration of 10 half-maximal units per ml except where noted (1 unit of PDGF or of FGF induces half-maximal stimulation of [$^3$H]thymidine uptake by 3T3 cells). Bovine pancreatic insulin and poly(I):poly(C) were from Sigma Chemical Co. (St. Louis, MO). B-TGF was from Biomedical Technologies, Inc. (Stoughton, MA). Platelet-poor plasma was from C. D. Stiles.

### Phosphatidylinositol and Protein Kinase Assays In Vitro

Immune complex PI and protein kinase assays were conducted as previously described (Whitman et al., 1985; Kaplan et al., 1986). PI and protein kinase assays were linear over the time of the assay.

### Radiolabeling of Cells and Immunoprecipitation

For $^{32}$P labeling, cells were washed three times with 2 ml of phosphate-free medium and then incubated at 37°C for 3 hr in 3 ml of phosphate-free medium supplemented with 2 mCi/ml of [$^{32}$P]orthophosphate, 2 mM glutamine, and 2% (v/v) platelet-poor plasma. For [$^{35}$S]methionine labeling, cells were labeled for 4 hr with 0.5 mCi/ml of [$^{35}$S]methionine in 1% platelet-poor plasma. For growth factor experiments, mitogens were added directly to the labeling medium. Cell lysates were prepared as described by Whitman et al. (1985) except for the addition of 400 μM ammonium ortho-vanadate and 10 mM EDTA, and the lysates were normalized for protein content. Immunoprecipitations were carried out as described by Whitman et al. (1985) with anti-phosphotyrosine antiserum (White et al., 1985) or with rabbit anti-polyoma T antiserum (Pallas et al., 1986). Protein-A Sepharose beads (Sigma) were added and the incubation continued for 1 hr at 4°C. Immunoprecipitates were then washed and analyzed by SDS–PAGE. PI kinase reactions were extracted and analyzed by TLC (Whitman et al., 1985).

### Peptide Mapping Analysis

Peptide mapping by limited proteolysis in SDS–PAGE gels was conducted as previously described (Schaffhausen and Benjamin, 1981). Proteins were resolved on cylindrical SDS–PAGE. The cylinder were placed on a polyacrylamide slab gel, and digestion was carried out by overlaying the gel with 2 ml of enzyme solution.

### Phosphoamino Acid Analysis

Phosphorylated proteins were isolated and digested with 6 N HCl as described previously (Schaffhausen et al., 1987). Electrophoresis was carried out on cellulose plates (Merck & Co., Inc.) in pyridine acetate (pH 3.1) at 1000 V. Phosphoamino acid standards (Sigma) were stained with 0.25% ninhydrin in n-butanol.

### Partial Purification of the 85 kd Protein and PI Kinase Activity

To elute the 85 kd protein and PI kinase activity from the anti-MTAg immunoprecipitates, immune complexes bound to Protein-A Sepharose beads were phosphorylated in vitro in the presence of 1 mM ammonium vanadate, 5 mM $MgCl_2$, and 20 μM [$\gamma$-$^{32}$P]ATP; the beads were washed as described by Whitman et al. (1985) and were incubated with 0.5% cholate, 0.2 mg/ml PI/phosphatidylserine (1:1), 0.1 M Tris (pH 7.5), 0.7 M NaCl, 1 mM ammonium vanadate, for 30 min at 4°C. The beads were then sedimented, and the supernatant was incubated with anti-P-tyr antiserum and the immunoprecipitates analyzed by SDS–PAGE.

For protocols involving wheat germ lectin chromatography, cell lysates (from 5 × 10$^6$ cells) were incubated with 100 μl of wheat germ lectin–agarose (Pharmacia) for 1 hr at 4°C. The precipitates were phosphorylated in vitro by incubation with 50 mM Tris (pH 7.4), 10 μM ATP, 1 mM ammonium vanadate, and 50 μCi [$\gamma$-$^{32}$P]ATP for 5 min at 25°C, were washed, and were eluted with 0.2 M N-acetylglucosamine, 0.5% β-octylglucoside, 0.1 M Tris (pH 7.5), for 45 min at 4°C. The eluate was diluted 10-fold in 0.2% β-octylglucoside, 20 mM Tris (pH 7.5), 1 mM ammonium vanadate, and was immunoprecipitated with anti-P-tyr antiserum and analyzed as described above.

To determine the specific activity of the 85 kd protein, the purification procedures described above were performed with [$^{35}$S]methionine-labeled cells. The activity of the PI kinase (mol phosphate incorporated per min) in the anti-P-tyr immunoprecipitates was determined by scintillation counting of the PIP reaction product from the PI kinase assays. The specific activity of the total [$^{35}$S]methionine-labeled cellular protein was determined by counting an aliquot of cell lysate and determining the dpm/μg. The eluted gel band containing the 85 kd protein was counted by scintillation counting.

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