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Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation

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The transforming protein of polyoma virus, middle T antigen, associates with the protein tyrosine kinase pp60$^{c\text{-}src}$, and analysis of mutants of middle T suggests that this complex plays an important role in transformation by polyoma. It has recently been reported that pp60$^{c\text{-}src}$ from polyma virus-transformed cells has enhanced tyrosine kinase activity *in vitro*. The data presented here confirm these findings and show that the enhanced kinase activity of pp60$^{c\text{-}src}$ is due to an increase in the V_{max} of the enzyme. Sucrose density gradient analysis demonstrates that only the form of pp60$^{c\text{-}src}$ which is bound to middle T antigen is activated. The difference in enzyme activity between pp60$^{c\text{-}src}$ from normal and middle T-transformed cells is more marked when the enzyme is prepared from lysates containing the phosphotyrosine protein phosphatase inhibitor, sodium orthovanadate. pp60$^{c\text{-}src}$ from middle T transformed cells is unaffected, but pp60$^{c\text{-}src}$ from normal cells has reduced kinase activity if dephosphorylation is prevented. The kinase activity of pp60$^{c\text{-}src}$ thus appears to be regulated by its degree of phosphorylation at tyrosine, and data are presented which support this hypothesis. pp60$^{c\text{-}src}$ is the first example of a protein tyrosine kinase whose activity is inhibited by phosphorylation at tyrosine. Middle T antigen may increase the kinase activity of pp60$^{c\text{-}src}$ by preventing phosphorylation at this regulatory site.

Key words: middle T antigen/phosphotyrosine/pp60$^{c\text{-}src}$/protein tyrosine kinase/sodium orthovanadate

Introduction

The transforming protein of polyoma virus, middle T antigen, is a membrane-associated phosphoprotein with an apparent mol. wt. of ~55 kd (for review, see Smith and Ely, 1983). Middle T antigen exists in a stable complex with a protein tyrosine kinase, pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983). pp60$^{c\text{-}src}$ is a plasma membrane protein (Courtneidge *et al.*, 1980) which is highly homologous to the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (Takeya and Hanafusa, 1983). A study of mutants of middle T antigen has shown that all mutants which retain the capacity to transform cells are also able to bind to pp60$^{c\text{-}src}$, whereas some non-transforming mutants are unable to form a stable complex (Courtneidge and Smith, 1984). These data suggest that complex formation is a necessary event for transformation to occur, although it may not be sufficient.

Neither the amount nor the turnover rate of pp60$^{c\text{-}src}$ in the cell is affected by middle T antigen binding (Bolen *et al.*, 1984; and unpublished observations). However, pp60$^{c\text{-}src}$ from polyoma-virus transformed cells phosphorylates substrates more efficiently in the *in vitro* kinase assay than pp60$^{c\text{-}src}$ from normal cells (Bolen *et al.*, 1984). This is only a property of pp60$^{c\text{-}src}$ from cells infected with transformation-competent mutants of middle T antigen, not pp60$^{c\text{-}src}$ from cells infected with non-transforming mutants (Bolen *et al.*, 1984). This correlation suggests that increased pp60$^{c\text{-}src}$ kinase activity is sufficient to transform cells. Yet it has also been reported that over-expression of pp60$^{c\text{-}src}$ in cells does not lead to transformation (Iba *et al.*, 1984; Parker *et al.*, 1984).

The function of pp60$^{c\text{-}src}$ is unknown. It is expressed in all cell types to approximately the same extent, except cells of neural origin, which have elevated levels (Cotton and Brugge, 1983; Sorge *et al.*, 1984). The expression of pp60$^{c\text{-}src}$ appears to be higher in terminally differentiated cells. To date no measurements of pp60$^{c\text{-}src}$ kinase activity, as opposed to concentration, have been made. The possibility exists therefore that different cell types with the same amount of pp60$^{c\text{-}src}$ will nevertheless contain different levels of pp60$^{c\text{-}src}$ kinase activity. Regulation of pp60$^{c\text{-}src}$ has not been described before, but the activity of other tyrosine kinases has been shown to be regulated by their degree of phosphorylation. For example, phosphorylation of the epidermal growth factor (EGF) receptor on threonine leads to decreased tyrosine kinase activity (Cochet *et al.*, 1984; Friedman *et al.*, 1984), whereas phosphorylation of the insulin receptor on tyrosine enhances activity (Rosen *et al.*, 1983).

Here, I show that the differences in kinase activity between pp60$^{c\text{-}src}$ from normal and polyoma virus-transformed cells is due to an increase in the specific activity of the enzyme. Only the pp60$^{c\text{-}src}$ which is complexed to middle T antigen has higher kinase activity. This difference in activity is more striking if the phosphotyrosine protein phosphatase inhibitor, sodium orthovanadate, is included in the lysis buffer. Under these conditions, pp60$^{c\text{-}src}$ from normal cells is a less efficient kinase, whereas pp60$^{c\text{-}src}$ from middle T transformed cells is unaffected. Thus the kinase activity of pp60$^{c\text{-}src}$ appears to be regulated by its degree of phosphorylation. This in turn suggests a mechanism by which middle T antigen may increase the kinase activity of pp60$^{c\text{-}src}$.

Results

The kinase activity of pp60$^{c\text{-}src}$ from normal and middle T antigen-transformed cells

Middle T antigen and pp60$^{c\text{-}src}$ co-exist in a stable complex in cell lysates. Immunoprecipitation of the complex with antibodies to either of the components followed by incubation with ATP leads to phosphorylation of the middle T antigen on tyrosine residues, as well as (under some circumstances) auto-phosphorylation of pp60$^{c\text{-}src}$ and/or phosphorylation of the specific antibody (Eckhart *et al.*, 1979; Schaffhausen and Benjamin, 1979; Smith *et al.*, 1979; Courtneidge and Smith, 1983, 1984; Bolen *et al.*, 1984). Since all mutants of middle T antigen which are capable of becoming phosphorylated in this *in vitro* kinase assay are complexed to pp60$^{c\text{-}src}$, it has been proposed that pp60$^{c\text{-}src}$ is responsible for phosphorylating middle T antigen (Courtneidge and Smith, 1984). The ability of middle T antigen to transform is also correlated with its ability to associate with

pp60$^{c\text{-}src}$, and it was proposed that middle T antigen might alter the enzyme activity or specificity of pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1984). The low abundance of pp60$^{c\text{-}src}$ makes it very difficult to purify the enzyme in order to study its properties. Therefore a method of assaying the enzyme quantitatively in immune complexes was devised.

Lysates of normal and polyoma virus-transformed cells were normalised with respect to protein concentration, and pp60$^{c\text{-}src}$ immunoprecipitated, using an antibody specific for its carboxy terminus (Courtneidge and Smith, 1984). The level of pp60$^{c\text{-}src}$ in the cell is unaffected by transformation by polyoma virus as judged by comparison of both c-*src* RNA and pp60$^{c\text{-}src}$ levels (Bolen *et al.*, 1984) and a comparison of the amount of pp60$^{c\text{-}src}$ in Rat1 and 3C3 cells labelled with [^{35}S]methionine for either 1 or 6 h (data not shown). These immune precipitates are therefore assumed to contain equal amounts of pp60$^{c\text{-}src}$. This enzyme preparation was assayed for its ability to phosphorylate exogenously added substrates as described in detail in Materials and methods. It was established that the reactions were linear for at least 10 min, were dependent upon the presence of pp60$^{c\text{-}src}$ and that the optimal divalent cation concentration was 5 mM (data not shown).

In the experiment presented in Figure 1, the effect on enzyme activity of varying the ATP concentration was determined. A Michaelis-Menten plot (Figure 1A) shows that both forms of the enzyme display typical saturation kinetics, but that there is a striking difference between the pp60$^{c\text{-}src}$ derived from normal cells and that derived from polyoma virus-transformed cells. The enzyme from polyoma virus-transformed cells was able to phosphorylate the substrate enolase at a much greater rate than the enzyme from normal cells. A Lineweaver-Burk plot of the same data (Figure 1B) revealed that in both cases, the apparent K_m for ATP was ~8 μM. However, the V_{max} of the enzyme from middle T-transformed cells was ~11 times higher than that from normal cells.

The previous experiment was performed using manganese as the divalent cation, yet within the cell all ATP would be in the form of MgATP. The same analysis was therefore performed using Mg instead of Mn. Again, both forms of the enzyme showed the same affinity for ATP (Table I), but the pp60$^{c\text{-}src}$ from polyoma virus-transformd cells had a much greater V_{max} than the pp60$^{c\text{-}src}$ from normal cells. It is interesting to note that the apparent K_m for ATP was much higher in the presence of Mg than Mn. In this respect pp60$^{c\text{-}src}$ is similar to the insulin receptor kinase (Nemenoff *et al.*, 1984). The effect of varying the concentration of the substrate enolase was also investigated (Table I). An apparent K_m for enolase of 1 μM was observed, and again the enzyme from polyoma virus-transformed cells was more efficient.

The results presented in Table I are representative analyses using the anti-c*src*.c antibody to immune precipitate pp60$^{c\text{-}src}$. These experiments have also been repeated using a monoclonal antibody to pp60$^{c\text{-}src}$ (Lipsich *et al.*, 1983) with the same results. In addition, using casein rather than enolase as substrate gives the same results. A similar comparison has also been made betwen NIH-3T3 cells and NIH-3T3 cells containing either a wild-type middle T antigen (101-3T3) or a non-transforming middle T antigen(NG59-3T3). pp60$^{c\text{-}src}$ from the 101-3T3 cells was ~20-fold more active than that from either normal or NG59-3T3 (data not shown). In summary, the binding of a transformation competent middle T antigen to pp60$^{c\text{-}src}$ did not change the K_m for substrates, but did result in a substantial (5- to 20-fold) increase in the apparent V_{max} of the enzyme.

The data obtained in these analyses have been used to derive conditions under which to assay the enzyme. The reaction mixture contains 20 mM Hepes pH 7.2, 250 μg/ml enolase, 20 μM ATP, 5 mM $MnCl_2$ and incubation is at 30°C for 4 min. All subsequent analyses presented in this paper use these conditions.

Only pp60$^{c\text{-}src}$ complexed to middle T antigen is a more efficient kinase

In previous reports (Courtneidge and Smith, 1983; Courtneidge *et al.*, 1984; Bolen *et al.*, 1984) it has been demonstrated that not all of pp60$^{c\text{-}src}$ in a polyoma virus-transformed cell is complexed to middle T antigen. These two forms of pp60$^{c\text{-}src}$, complexed and free, can be separated on a sucrose density gradient.

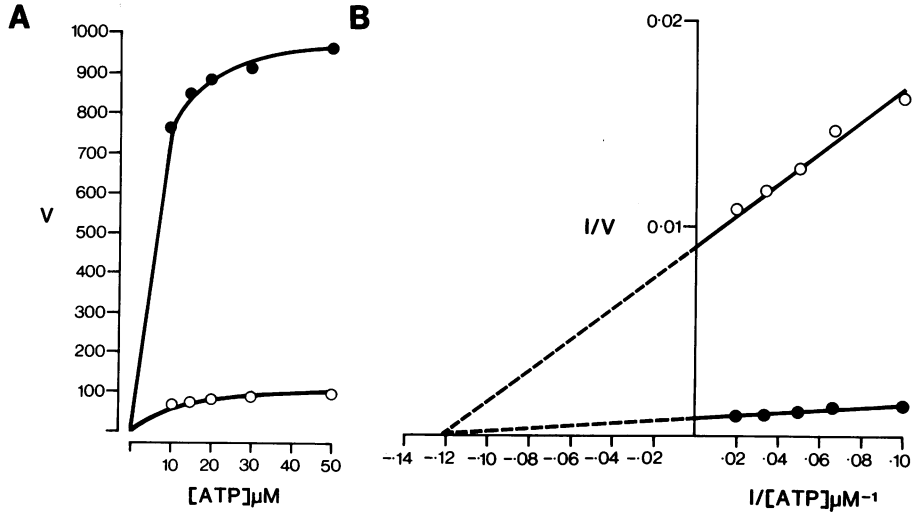

Fig. 1. The kinase activity of pp60$^{c\text{-}src}$ from normal and middle T antigen-transformed cells. Lysates of Rat1 and polyoma virus-transformed Rat1 (3C3 cells) were prepared as described in Materials and methods. The lysis buffer included 100 μM sodium orthovanadate. Kinase reactions were performed as indicated in a buffer containing 20 mM Hepes pH 7.2, 5 mM $MnCl_2$, 250 μg/ml enolase, and the indicated concentration of ATP. **Panel A:** the *x* axis denotes the concentration of ATP and the *y* axis the rate of the reaction expressed as femtomoles phosphate transferred to enolase/min/mg of lysate. Open circles, pp60$^{c\text{-}src}$ from Rat1 cells. Closed circles, pp60$^{c\text{-}src}$ from 3C3 cells. **Panel B:** a double reciprocal plot of the data from **Panel A.** Open circles, pp60$^{c\text{-}src}$ from Rat1 cells. Closed circles, pp60$^{c\text{-}src}$ from 3C3 cells.

Table I. The kinase activity of pp60$^{c\text{-}src}$

Substrate		Source of enzyme		V_{max} 3C3
		Rat1	3C3	V_{max} Rat1
MnATP	K_m [a]	8	8	
	V_{max} [b]	109	1190	10.9
MgATP	K_m	42	42	
	V_{max}	128	1429	11.1
Enolase	K_m	1	1	
	V_{max}	70	435	6.2

[a] K_ms are expressed as micromolar.
[b] V_{max}s are expressed as femtomoles of phosphate transferred to enolase/min/mg of cell lysate.
The figures provided are from three representative experiments. In other experiments the values for K_m and V_{max} were within ± 50% of those shown here. The stimulation of kinase activity in 3C3 cells compared with Rat1 cells is always between 5- and 20-fold.

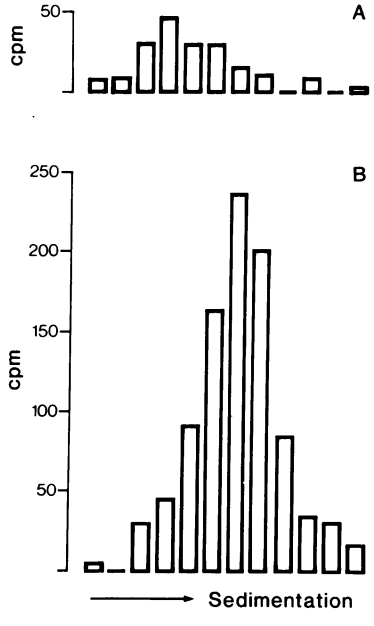

Fig. 2. Sucrose density gradient analysis of pp60$^{c\text{-}src}$ from normal and middle T antigen-transformed cells. Lysates of Rat1 and 3C3 cells were prepared and fractionated on 5–20% sucrose density gradients as described previously (Courtneidge and Smith, 1983), except that both the lysis buffer and the gradients contained 100 μM sodium orthovanadate. Sedimentation was for 6 h at 54 000 r.p.m. in an SW55 rotor. 24 fractions were collected and pp60$^{c\text{-}src}$ immune precipitated from alternate fractions with anti-c*src*.c. Kinase assays were performed as described. Shown is the amount of radioactivity (in c.p.m.) in enolase after a 4 min reaction.
Panel A: representation of a gradient containing a Rat1 cell lysate.
Panel B: representation of a gradient containing a 3C3 cell lysate.

To examine whether all of the pp60$^{c\text{-}src}$ in a polyoma virus-transformed cell has increased kinase activity, or only that form of pp60$^{c\text{-}src}$ complexed to middle T antigen, the following experiment was performed. Lysates of normal and polyoma virus-transformed Rat1 cells were sedimented on sucrose density gradients, pp60$^{c\text{-}src}$ immune precipitated and its kinase activity determined. The results are presented in Figure 2. Panel A shows that the kinase activity associated with the pp60$^{c\text{-}src}$ in normal Rat1 cells sedimented with an approximate mol. wt. of 60 kd as described previously (Courtneidge and Smith, 1983). In contrast, the bulk of the pp60$^{c\text{-}src}$ kinase activity in a polyoma virus-transformed cell sedimented at ~220 kd (Panel B). This is the region of the gradient which contains the middle T:pp60$^{c\text{-}src}$ complex.

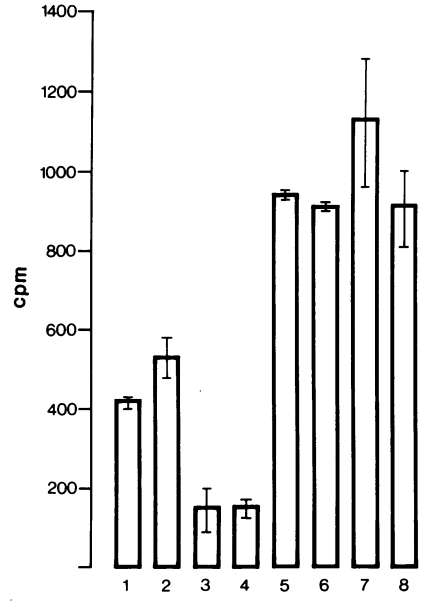

Fig. 3. The effect of sodium orthovanadate on the kinase activity of pp60$^{c\text{-}src}$. Lysates were prepared from Rat1 cells **(lanes 1–4)**, and 3C3 cells **(lanes 5–8)** either without **(lanes 1, 2, 5** and **6)** or with **(lanes 3, 4, 7** and **8)** 100 μM sodium orthovanadate. pp60$^{c\text{-}src}$ was immune precipitated from these lysates and prepared for kinase assay as described. In some cases, the enzyme preparation was resuspended into a kinase buffer which contained 100 μM sodium orthovanadate, and incubated for 15 min on ice prior to addition of ATP **(lanes 2, 4, 6** and **8)**. Depicted are the number of counts in enolase at the end of 4 min kinase reactions. The reactions were carried out in triplicate and error bars show the range of values obtained.

The fact that the region of the gradient of polyoma virus-transformed cell lysates containing free pp60$^{c\text{-}src}$ contained only very low levels of kinase activity suggests that it is only the complexed form of pp60$^{c\text{-}src}$ which is a more active enzyme. This in turn implies that middle T acts directly on pp60$^{c\text{-}src}$ to increase its kinase activity.

The effect of a phosphotyrosine protein phosphatase inhibitor on the kinase activity of pp60$^{c\text{-}src}$

All of the lysates used in the analyses described in the previous sections were prepared in buffers containing inhibitors to prevent proteolysis and dephosphorylation. Recent reports have shown that lysing cells in the presence of the phosphotyrosine-specific phosphatase inhibitor used in this study (sodium orthovanadate) allows the detection of a more active form of the closely related tyrosine kinase, pp60$^{v\text{-}src}$ (Brown and Gordon, 1984; Collett *et al.*, 1984). For this reason, I decided to examine whether the sodium orthovanadate had any effect on pp60$^{c\text{-}src}$. Both normal and polyoma virus-transformed Rat1 cells were lysed in the presence or absence of 100 μM sodium orthovanadate and kinase assays carried out. The results are shown in Figure 3. pp60$^{c\text{-}src}$ from polyoma virus-transformed cells was not significantly affected by orthovanadate, whether it was present during the immune precipitation (lane 7) or the kinase assay (lanes 6 and 8). However, the inhibitor did have an effect on pp60$^{c\text{-}src}$ from normal cells; lysates made with orthovanadate (lanes 3 and 4) had 3- to 5-fold less pp60$^{c\text{-}src}$ kinase activity than lysates made without orthovanadate (lanes 1 and 2). The orthovanadate had to be present from the moment the cells were lysed, and did not inhibit when added just prior to the kinase assay (lanes 2 and 4). This analysis has also been undertaken using a monoclonal antibody to pp60$^{c\text{-}src}$ to immune precipitate the protein, and identical results obtained (data not shown).

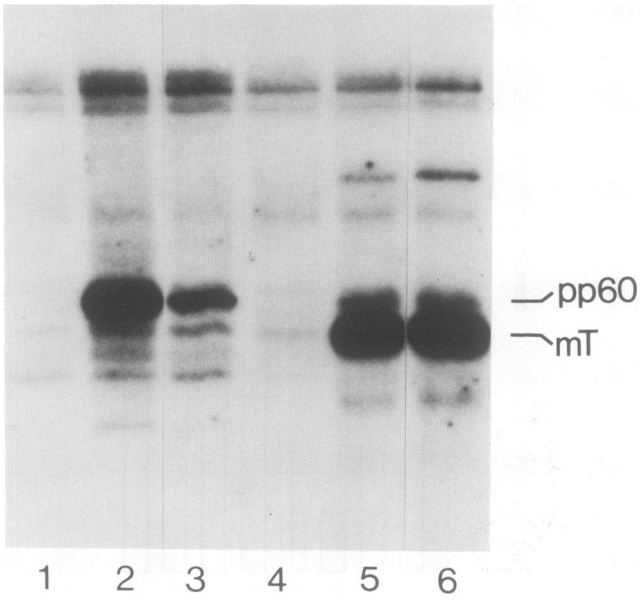

Fig. 4. The effect of sodium orthovanadate on the autophosphorylation of pp60$^{c\text{-}src}$. Lysates were prepared from Rat1 cells **(lanes 1–3)** and 3C3 cells **(lanes 4–6)** either without **(lanes 1, 2, 4** and **5)** or with **(lanes 3** and **6)** sodium orthovanadate. Immune precipitates were prepared using a monoclonal antibody to pp60$^{c\text{-}src}$ **(lanes 2, 3, 5** and **6)** or without specific antibody **(lanes 1** and **4)**. Phosphorylation reactions were carried out in a buffer containing 20 mM Hepes pH 7.2, 5 mM $MnCl_2$, and 1 μM ATP containing 10 μCi [γ-^{32}P]ATP for 4 min at 30°C. The reaction was terminated by the addition of gel loading buffer and the products examined by SDS-gel electrophoresis and autoradiography.

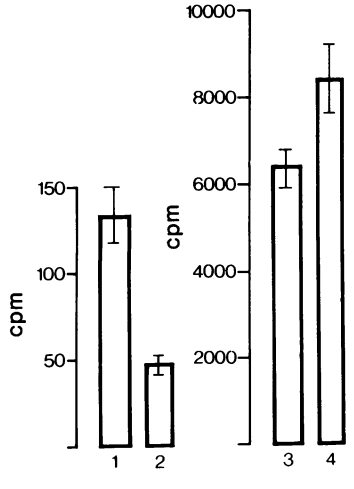

Fig. 5. The effect of sodium orthovanadate on the kinase activities of pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$. Rous sarcoma virus-transformed BALB/3T3 cells (SR.1) were lysed in either the absence **(lanes 1** and **3)** or presence **(lanes 2** and **4)** of 100 μM sodium orthovanadate. Immune precipitates were made with either anti-c*src*.c **(lanes 1** and **2)** or anti-v*src*.c **(lanes 3** and **4)** antibodies, and kinase assays performed as described. The figure shows the incorporation of label into enolase during a 4 min reaction. Error bars show the range of values obtained in triplicate reactions.

The change in pp60$^{c\text{-}src}$ kinase activity was also reflected in the ability of the enzyme to autophosphorylate. Figure 4 shows that pp60$^{c\text{-}src}$ derived from an orthovanadate-treated lysate of Rat1 (lane 3) showed much less autophosphorylation activity than pp60$^{c\text{-}src}$ from an untreated lysate (lane 2). In contrast, pp60$^{c\text{-}src}$ from polyoma virus-transformed cells isolated in the presence and absence of orthovanadate autophosphorylated to the same extent (lanes 5 and 6).

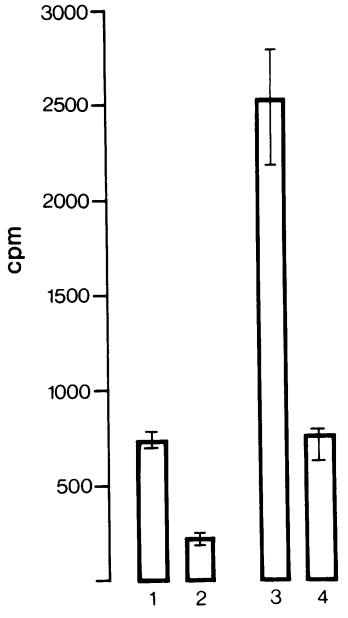

Fig. 6. The effect of sodium orthovanadate on the kinase activity of pp60$^{c\text{-}src}$ derived from cells containing elevated levels of pp60$^{c\text{-}src}$. Rat2 cells **(lanes 1** and **2)** and RC cells which contain 10 times the normal level of pp60$^{c\text{-}src}$ (Parker *et al.*, 1984) **(lanes 3** and **4)** were lysed in either the absence **(lanes 1** and **3)** or presence **(lanes 2** and **4)** of sodium orthovanadate, and kinase assays performed as described. The figure shows the incorporation of label into enolase during a 4 min reaction. Error bars show the range of values obtained in triplicates.

These results are in contrast to the reported effect of phosphatase inhibition on pp60$^{v\text{-}src}$ (Brown and Gordon, 1984; Collett *et al.*, 1984). It seemed important therefore to measure the kinase activities of pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ derived from the same lysate. To do this an RSV-transformed cell line, SR.1, was lysed in the presence or absence of orthovanadate. pp60$^{c\text{-}src}$ was immune precipitated using the anti-c*src*.c antibody, and pp60$^{v\text{-}src}$ was immune precipitated using an antibody which recognises the carboxy-terminal six amino acids of pp60$^{v\text{-}src}$ (anti-v*src*.c). As shown in Figure 5, the kinase activity of pp60$^{v\text{-}src}$ was modestly increased in lysates containing orthovanadate (lanes 3 and 4), in agreement with previous reports (Brown and Gordon, 1984; Collett *et al.*, 1984). However, the pp60$^{c\text{-}src}$ kinase activity was lower in lysates containing orthovanadate (lane 2), than in untreated lysates (lane 1). So even within the same cell lysate, pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ were affected differently by orthovanadate.

Recently, cells containing at least 10 times the normal level of pp60$^{c\text{-}src}$ (which are phenotypically untransformed) have become available (Iba *et al.*, 1984; Parker *et al.*, 1984). I measured the levels of pp60$^{c\text{-}src}$ kinase activity in one such cell line, RC, and the cell line from which it was derived, Rat2 (Figure 6). In both cell lines the pp60$^{c\text{-}src}$-associated kinase activity was markedly inhibited when lysates were prepared with sodium orthovanadate. To ensure that the orthovanadate was not affecting the level of pp60$^{c\text{-}src}$ in the cell lysate, both Rat2 and RC cells were labelled with [^{35}S]methionine and the amount of immunoprecipitable pp60$^{c\text{-}src}$ measured. The same amount of pp60$^{c\text{-}src}$ was recovered from lysates with and without sodium orthovanadate (Figure 7).

The fact that sodium orthovanadate did not change the level of pp60$^{c\text{-}src}$ in cell lysates, that it did not affect the enzyme once

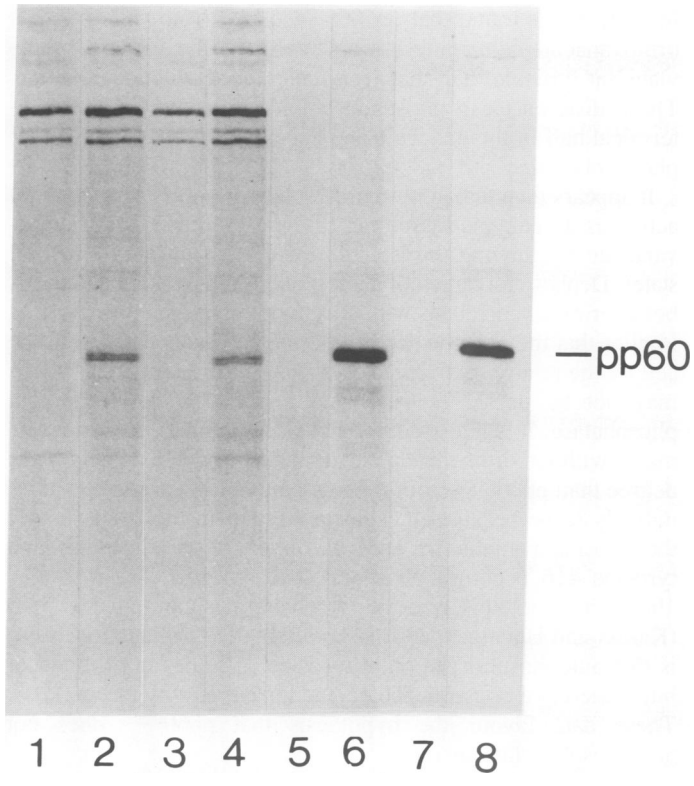

Fig. 7. An analysis of the amount of pp60$^{c\text{-}src}$ in lysates containing sodium orthovanadate. Rat2 **(lanes 1–4)** and RC **(lanes 5–8)** cells were labelled with [^{35}S]methionine for 2 h at 37°C and lysed in the absence **(lanes 1, 2, 5** and **6)** or presence **(lanes 3, 4, 7** and **8)** of sodium orthovanadate. Immune precipitates were made with either non-specific antibody **(lanes 1, 3, 5** and **7)** or a monoclonal antibody to pp60$^{c\text{-}src}$ **(lanes 2, 4, 6** and **8)** as described previously (Courtneidge and Smith, 1983), and analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

Table II. The effect of sodium orthovanadate on the phosphorylation of pp60$^{c\text{-}src}$

	Lysates made	
	Without orthovanadate	With orthovanadate
^{32}P in pp60$^{c\text{-}src}$	1[a]	1.25
$\frac{^{32}\text{P in COOH terminus}}{^{32}\text{P in NH2 terminus}}$	0.95	1.43
$\frac{^{32}\text{P in P-Tyr}}{^{32}\text{P in P-Ser}}$	0.49	0.75

pp60$^{c\text{-}src}$ was immune precipitated from parallel [^{32}P]orthophosphate-labelled cultures of RC cells which were identical except that the lysis buffer for one contained 100 μM sodium orthovanadate. Other experiments analysing the pp60$^{c\text{-}src}$ from Rat1 cells gave the same results.
[a]The amount of ^{32}P in pp60$^{c\text{-}src}$ has been normalised to a value of 1 for the lysates without orthovanadate.

immunoprecipitated, and that it had to be present from the moment the cells were lysed to be effective, suggested that it was exerting its effect by inhibiting a phosphotyrosine-specific protein phosphatase. If this were the case, one would expect there to be a difference in phosphorylation of pp60$^{c\text{-}src}$ isolated from lysates with and without orthovanadate. To examine this, pp60$^{c\text{-}src}$ was immune precipitated from [^{32}P]orthophosphate-labelled cells. RC cells (which contain elevated levels of pp60$^{c\text{-}src}$) were used in the first instance in order to generate large quantitites of pp60$^{c\text{-}src}$. However many of these experiments have been repeated with the pp60$^{c\text{-}src}$ of Rat1 cells with identical results. Table II summarises the results of analyses of pp60$^{c\text{-}src}$ from orthovanadate-treated and untreated lysates. pp60$^{c\text{-}src}$ from vanadate-treated cultures consistently contained more ^{32}P than pp60$^{c\text{-}src}$ isolated from parallel untreated cultures. One-dimensional peptide mapping using *Staphylococcus aureus* V8 protease was used to divide pp60$^{c\text{-}src}$ into the amino-terminal (predominantly phosphoserine-containing) and carboxy-terminal (predominantly phosphotyrosine-containing) fragments. An analysis of the phosphate content of the two fragments showed that the increased phosphorylation of pp60$^{c\text{-}src}$ from orthovanadate-treated cultures was confined to the carboxy-terminal fragment. Furthermore, phosphoamino acid analysis showed that pp60$^{c\text{-}src}$ from orthovanadate-treated lysates contained a higher ratio of phosphotyrosine to phosphoserine that pp60$^{c\text{-}src}$ from untreated lysates.

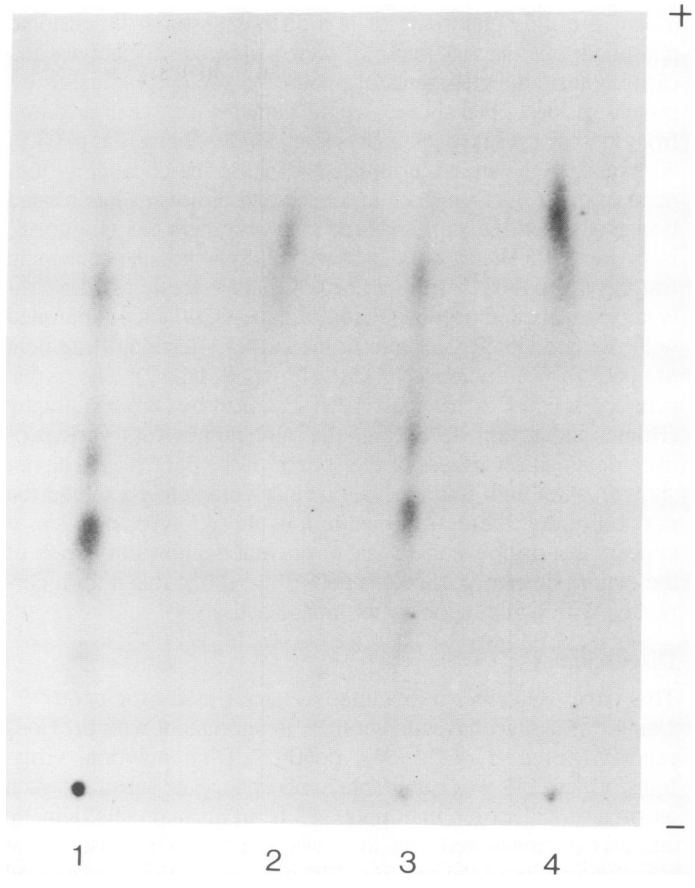

Fig. 8. An analysis of the phosphotryptic peptides of pp60$^{c\text{-}src}$ derived from cells lysed with and without sodium orthovanadate. RC cells were labelled with [^{32}P]orthophosphate and lysed in either the presence or absence of sodium orthovanadate. pp60$^{c\text{-}src}$ was immune precipitated, and hydrolysis with V8 protease used to isolate the 34-kd amino-terminal and 26-kd carboxy-terminal domains. The separated fragments were then oxidised with performic acid, digested with trypsin and the resultant peptides fractionated by electrophoresis at pH 8.9. **Lanes: 1,** amino-terminal domain of pp60$^{c\text{-}src}$ from lysates without orthovanadate; **2,** carboxy-terminal domain of pp60$^{c\text{-}src}$ from lysates without orthovanadate; **3,** amino-terminal domain of pp60$^{c\text{-}src}$ from lysates with orthovanadate; **4,** carboxy-terminal domain of pp60$^{c\text{-}src}$ from lysates with orthovanadate.

To date, only one phosphotyrosine site has been identified on pp60$^{c\text{-}src}$. This is in the carboxy terminus but does not correspond to the phosphotyrosine site of pp60$^{v\text{-}src}$ (Karess and Hanafusa, 1981; Smart *et al.*, 1981). To determine whether the increased phosphorylation observed in the presence of orthovana-

date was at this major site or at a different site, 1-dimensional phosphotryptic maps of pp60$^{c\text{-}src}$ were produced. The amino- and carboxy-terminal fragments of pp60$^{c\text{-}src}$ were derived using V8 protease as described above. Tryptic peptides were then prepared from these fragments, and analysed by electrophoresis at pH 8.9. As expected, the amino-terminal, phosphoserine-containing fragment of pp60$^{c\text{-}src}$ from both untreated and orthovanadate-treated lysates showed the same pattern of tryptic peptides (Figure 8, lanes 1 and 3). The carboxy-terminal, phosphotyrosine-containing fragment of pp60$^{c\text{-}src}$ from an orthovanadate-treated lysate contained one labelled peptide (Figure 8, lane 4), which co-migrated with the one labelled peptide in the carboxy-terminal fragment of pp60$^{c\text{-}src}$ from untreated lysates (Figure 8, lane 2). The tryptic peptides of pp60$^{c\text{-}src}$ have also been separated by chromatography (Hunter and Sefton, 1980), and the same number of tryptic peptides obtained regardless of the source of the pp60$^{c\text{-}src}$ (data not shown). Although this analysis cannot completely exclude the possibility that there are other minor phosphotyrosine sites, it appears that orthovanadate acts to prevent dephosphorylation of the major phosphotyrosine of pp60$^{c\text{-}src}$, and that this in turn correlates with a reduction in its kinase activity.

Discussion

This paper describes a quantitative kinase assay for pp60$^{c\text{-}src}$. Using this assay I have shown that, in agreement with previous reports (Bolen *et al.*, 1984), pp60$^{c\text{-}src}$ from polyoma virus-transformed cells was better able to phosphorylate substrates such as enolase and casein than pp60$^{c\text{-}src}$ from normal cells. Kinetic analysis demonstrated that this was due to an increase in the specific activity of the enzyme. Since these reactions are carried out at ATP concentrations in the physiological range, they imply that pp60$^{c\text{-}src}$ complexed to middle T is also able to phosphorylate substrates at a greater rate *in vivo*. Not all of the pp60$^{c\text{-}src}$ in a middle T antigen-transformed cell appears to be complexed to middle T, but only the form of pp60$^{c\text{-}src}$ which is complexed to middle T antigen has enhanced tyrosine kinase activity.

How might middle T antigen increase the kinase activity of pp60$^{c\text{-}src}$? Two possible ways come to mind. Firstly, the binding of middle T antigen might cause a conformational change in pp60$^{c\text{-}src}$ leading to enhanced activity. Secondly, middle T antigen might mask a regulatory site on pp60$^{c\text{-}src}$ such that the enzyme activity can no longer be modulated within the cell. Since pp60$^{c\text{-}src}$ is a phosphoprotein, one obvious way in which it could be regulated is by degree of phosphorylation. Indeed it has been shown that the closely related enzyme pp60$^{v\text{-}src}$ exists in two forms – one of which is more highly phosphorylated at tyrosine and has greater kinase activity than the other (Collett *et al.*, 1983; Purchio *et al.*, 1983; Brown and Gordon, 1984; Collett *et al.*, 1984), and is only evident if the lysis buffer contains either sodium orthovanadate or high concentrations of ATP (Brown and Gordon, 1984; Collett *et al.*, 1984). A similar analysis of the effect of sodium orthovanadate on pp60$^{c\text{-}src}$ showed that the kinase activity of pp60$^{c\text{-}src}$ from polyoma virus-transformed cells was unaffected by orthovanadate, but pp60$^{c\text{-}src}$ from Rat1 cells displayed 3- to 5-fold less activity if lysates contained orthovanadate.

Vanadate is a naturally occurring compound which has many effects in cells (Ramasarma and Crane, 1981), including inhibiting the NaK-ATPase (Cantley *et al.*, 1978), RNase (Lindquist *et al.*, 1973) and phosphotyrosine-specific protein phosphatases (Swarup *et al.*, 1982). Because pp60$^{c\text{-}src}$ is a phosphotyrosine-containing protein it seemed most likely that the orthovanadate effect was due to its inhibition of dephosphorylation of pp60$^{c\text{-}src}$ at tyrosine. Indeed, it was found that pp60$^{c\text{-}src}$ isolated from cells lysed in orthovanadate-containing buffers was more heavily phosphorylated at tyrosine than that from cells lysed without inhibitor. These differences in phosphorylation resided in the carboxy-terminal half of the molecule and appeared to involve the major phosphotyrosine site.

It appears then that in normal fibroblasts, pp60$^{c\text{-}src}$ is not very active as an enzyme (assuming that the presence of the orthovanadate is allowing the isolation of the enzyme in its natural state). Dephosphorylation of a phosphotyrosine residue in the carboxy terminus correlates with an activation of the enzyme. This implies that the enzyme activity of pp60$^{c\text{-}src}$ is controlled in the cell by the relative activities of a tyrosine kinase (which may or may not be pp60$^{c\text{-}src}$ itself) and a phosphotyrosine protein phosphatase. It is interesting to note that pp60$^{c\text{-}src}$ from a lysate made without orthovanadate autophosphorylated to a greater degree than pp60$^{c\text{-}src}$ from a lysate containing vanadate. This is unlikely to be because phosphatase action in the lysate makes the tyrosine available for authophosphorylation, since although tyrosine 416 becomes phosphorylated *in vitro* (Smart *et al.*, 1981), it is not this tyrosine which is phosphorylated *in vivo* (Karess and Hanafusa, 1981). An alternative explanation, then, is that autophosphorylation at tyrosine 416 does not influence, but is merely a consequence of, the kinase activity of pp60$^{c\text{-}src}$. These data favour the hypothesis that pp60$^{c\text{-}src}$ does not autophosphorylate *in vivo*.

The results presented here show that introduced and over-expressed chicken pp60$^{c\text{-}src}$ has little kinase activity and is regulated in the same way as the endogenous rat pp60$^{c\text{-}src}$. This may explain the observation that cells which over-express pp60$^{c\text{-}src}$ are phenotypically normal (Iba *et al.*, 1984; Parker *et al.*, 1984). In keeping with this is the fact that chicken cells containing elevated levels of pp60$^{c\text{-}src}$ do not contain elevated levels of phosphotyrosine in cell proteins (Iba *et al.*, 1984).

The kinase activity of pp60$^{v\text{-}src}$, unlike that of pp60$^{c\text{-}src}$, is stimulated by orthovanadate. This could arise because of the inability of cellular enzymes to phosphorylate pp60$^{v\text{-}src}$ at a regulatory site, either because of sequence divergence or conformational differences from pp60$^{c\text{-}src}$. This would then allow pp60$^{v\text{-}src}$ to be active in cells when pp60$^{c\text{-}src}$ is not, which may be an important feature in transformation by *src*.

There are other examples, besides pp60$^{v\text{-}src}$, of tyrosine kinases which are more active when phosphorylated on tyrosine, including the insulin receptor (Rosen *et al.*, 1983) and P140$^{gag\text{-}fps}$, the transforming protein of Fujinami sarcoma virus (Weinmaster *et al.*, 1984). However, this is the first example of a tyrosine kinase whose activity is negatively regulated by tyrosine phosphorylation. There is however a precedent for such negative control: the serine-specific myosin light chain kinase is less active when phosphorylated at serine by the cyclic AMP-dependent protein kinase than in the absence of serine phosphorylation (Conti and Adelstein, 1981).

What can these experiments tell us about transformation by middle T antigen? Middle T may activate pp60$^{c\text{-}src}$ by binding to it in such a way that it prevents or lowers the degree of phosphorylation at the regulatory site. [This is difficult to examine directly. Simply analysing pp60$^{c\text{-}src}$ from polyoma virus transformed cells does not give clear-cut results (unpublished observations), presumably because not all of the pp60$^{c\text{-}src}$ is complexed to middle T antigen.] Transformation could than result because of inappropriate expression of the pp60$^{c\text{-}src}$ kinase activity, rather than phosphorylation of inappropriate substrates. If this is the case, polyoma virus-transformed cells will be an

excellent system for identifying the normal substrates of pp60$^{c\text{-}src}$.

It seems improbable that the kinase activity of pp60$^{c\text{-}src}$ is inhibited in all normal cells at all times. Rather, it seems more likely that middle T antigen disrupts the balance between kinase and phosphatase action which regulates pp60$^{c\text{-}src}$. Future experiments will seek to study this regulation in more detail, as well as to examine the kinase activity of pp60$^{c\text{-}src}$ as a result of normal stimuli, e.g., growth factors, in different cell types, and at different stages of the cell cycle.

Materials and methods

Reagents

All chemicals used were Analar or the purest grade available. Sodium orthovanadate was purchased from Aldrich and prepared as described in Brown and Gordon (1984).

Antibodies

The monoclonal antibody to pp60^{src} (Lipsich *et al.*, 1983) was obtained from Dr. Joan Brugge. The anti-peptide antibodies to both pp60$^{v\text{-}src}$ (anti-v*src*.c) and pp60$^{c\text{-}src}$ (anti-c*src*.c) have been described before (Courtneidge and Smith, 1984).

Cells

Cells used were: Rat1 cells and a derivative containing middle T antigen (3C3) obtained from Dr. Barry Ely; NIH-3T3 cells and derivatives containing wild-type middle T antigen (101-3T3) and the middle T antigen encoded by NG59 polyoma virus (NG59-3T3) obtained from Drs. Seng Cheng and Bill Markland; Rat2 cells and a derivative containing amplified levels of pp60$^{c\text{-}src}$ (RC) obtained from Dr. Richard Parker, and BALB/3T3 cells and a derivative containing Rous sarcoma virus (SR.1) obtained from Dr. John Wyke.

Methods

Methods for labelling of cells, immunoprecipitation of proteins, middle T kinase assays, sucrose density gradients, SDS-polyacrylamide gel electrophoresis, and one-dimensional peptide mapping have all been described previously (Courtneidge and Smith, 1983, 1984; Courtneidge *et al.*, 1984).

Lysates were prepared by washing monolayers of cells with, and scraping them into, ice-cold TBS (20 mM Tris, pH 7.5, 150 mM NaCl). Cells were pelleted by centrifugation at 1000 *g* for 5 min in the cold, and then resuspended into lysis buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% aprotinin). (Where indicated in the text, both the TBS and lysis buffer also contained 100 μM sodium orthovanadate.) Following incubation on ice for 15 min, insoluble material was removed by centrifugation at 14 000 *g* for 15 min in the cold. The protein concentration of the supernatants was determined (Bradford, 1976), parallel lysates of normal and middle T transformed cells adjusted to the same protein concentration (between 500 and 2000 μg/ml), and stored in liquid nitrogen until use.

Quantitative kinase assays were performed in the following way: immune precipitates of pp60 were prepared as described previously (Courtneidge and Smith, 1984) and washed twice in RIPA buffer and once in TBS. The kinase reaction was performed in a buffer containing 20 mM Hepes pH 7.2, 5 mM $MgCl_2$ or 5 mM $MnCl_2$, enolase (heat- and acid-denatured as described in Cooper *et al.*, 1984) and [γ-^{32}P]ATP (4–20 Ci/mmol). Except where noted in the text, the divalent cation was Mn, the enolase concentration was 250 μg/ml, and the ATP concentration was 20 μM. Reactions were carried out for 4 min at 30°C, were stopped by the addition of 4 times gel loading buffer (160 mM Tris pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol) and then heated to 70°C for 5 min. The products of the reaction were separated on an SDS-polyacrylamide gel and the enolase visualised by staining with Coomassie brilliant blue. The region of the gel containing the enolase was excised and counted in a scintillation counter to determine the extent of the reaction. As a negative control, immune precipitates made with anti-peptide antibody pre-blocked with specific peptide were also analysed, and these values have been subtracted from the experimental values. Results are expressed as femtomoles of phosphate transferred to enolase/min/mg of starting extract. The error bars show the range of values obtained in triplicate experiments.

Phosphoamino acid analysis was carried out as described in Cooper *et al.* (1983). Tryptic peptides were prepared as described in Karess and Hanafusa (1981), and analysed by electrophoresis in 1% ammonium carbonate pH 8.9 and/or by chromatography in butanol:pyridine:acetic acid:water (75:50:15:60) as described in Hunter and Sefton (1980).

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