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Enzymatic activation of Fujinami sarcoma virus *gag–fps* transforming proteins by autophosphorylation at tyrosine

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Site-directed mutagenesis of the Fujinami sarcoma virus (FSV) genome has suggested that Tyr 1073 of the P130$^{gag-fps}$ protein-tyrosine kinase is a regulatory site. To investigate directly the ability of tyrosine phosphorylation to affect P130$^{gag-fps}$ kinase activity, the phosphotyrosyl phosphatase inhibitor orthovanadate and partially purified phosphotyrosyl phosphatases were used to manipulate the stoichiometry of P130$^{gag-fps}$ phosphorylation. Phosphorylation of P130$^{gag-fps}$ at Tyr 1073 correlated with enhanced kinase activity. The thermolabile phosphorylation, kinase activity and transforming ability of P140$^{gag-fps}$ encoded by a temperature-sensitive (*ts*)FSV variant were restored at the non-permissive temperature for transformation by incubation of infected cells with orthovanadate. In this case tyrosine phosphorylation can apparently functionally reactivate a conditionally defective v-*fps* kinase activity. These data suggest that reversible autophosphorylation at a conserved tyrosine within the v-*fps* kinase domain is a positive regulator of enzymatic activity and biological function. Phenotypic suppression of the *ts*FSV genetic defect by orthovanadate emphasizes the potential importance of phosphotyrosyl phosphatases in antagonizing tyrosine kinase action. It is suggested that autophosphorylation may constitute a molecular switch by which some protein-tyrosine kinases are activated.

Key words: tyrosine kinase/autophosphorylation/neoplastic transformation/phenotypic suppression

Introduction

A majority of retroviral oncogenes encode protein-tyrosine kinases whose normal counterparts are either transmembrane growth factor receptors or cytoplasmic proteins of unknown function (Hunter and Cooper, 1985). Tyrosine kinases are generally capable of autophosphorylation (Pawson *et al.*, 1980; Rosen *et al.*, 1983; Hunter and Cooper, 1985), and can also serve as substrates for cellular serine/threonine-specific protein kinases (Hunter *et al.*, 1984; Gould *et al.*, 1985). The post-translational modification of tyrosine kinases by reversible phosphorylation may therefore represent a critical component in their biochemical regulation.

The transforming protein of Fujinami avian sarcoma virus (FSV), P130$^{gag-fps}$, becomes autophosphorylated at several tyrosine residues within its C-terminal v-*fps* domain (Weinmaster *et al.*, 1983, 1984). A major site of phosphorylation at residue 1073 in the P130$^{gag-fps}$ catalytic domain corresponds to a tyrosine that is invariant in all protein-tyrosine kinases (Hunter and Cooper, 1985). The substitution of this residue with phenylalanine, serine, threonine or glycine using oligonucleotide-directed mutagenesis of the FSV genome results in decreased P130$^{gag-fps}$ kinase activity and diminished oncogenic potential (Weinmaster *et al.*, 1984; Weinmaster and Pawson, 1986). These results have suggested that tyrosine phosphorylation at this site stimulates P130$^{gag-fps}$ enzymatic activity, and thereby contributes to its transforming capacity. However, an alternative interpretation of these data might be that Tyr 1073 is required for optimal enzymatic function regardless of its phosphorylation state.

To address this issue we have used reagents that can manipulate the occupancy of tyrosine sites by phosphate. Orthovanadate is an efficient inhibitor of phosphotyrosyl phosphatases both *in vitro* and also apparently *in vivo* (Swarup *et al.*, 1982; Nelson and Branton, 1984; Klarlund, 1985). We have previously reported the isolation and partial purification of orthovanadate-sensitive phosphotyrosyl phosphatases from chicken embryo fibroblasts (Nelson and Branton, 1984). These enzymes act effectively on phosphotyrosine, but have little activity against substrates containing phosphoserine.

Application of orthovanadate to Rous sarcoma virus (RSV)-transformed cells results in the accumulation of a highly phosphorylated form of p60$^{v\text{-}src}$ that contains a novel site of N-terminal tyrosine phosphorylation and displays increased kinase activity (Brown and Gordon, 1984; Collett *et al.*, 1984; Resh and Erikson, 1985). In contrast orthovanadate has an inhibitory effect on p60$^{c\text{-}src}$ activity, apparently by increasing the occupancy of a C-terminal tyrosine (Tyr 527) whose phosphorylation may depress kinase activity and which is absent from p60$^{v\text{-}src}$ (Courtneidge, 1985; Coussens *et al.*, 1985; Cooper *et al.*, 1986). The addition of orthovanadate to mammalian fibroblasts mimics some aspects of the neoplastic phenotype induced by transforming tyrosine kinases, apparently as a consequence of increased tyrosine phosphorylation of cellular proteins (Klarlund, 1985). Here we have used orthovanadate and phosphotyrosyl phosphatases to raise or lower the stoichiometry of tyrosine phosphorylation of FSV-transforming proteins. We find that increased tyrosine phosphorylation of P130$^{gag-fps}$ correlates with enhanced kinase activity and is sufficient to reactivate a defective kinase encoded by an FSV variant which is temperature-sensitive (*ts*) for transformation. The implications of these results for the functional regulation of protein-tyrosine kinases are discussed.

Results

Enhanced tyrosine phosphorylation of P130$^{gag-fps}$ in the presence of orthovanadate

FSV-transformed rat-2 cells were incubated for 4 h in medium containing [^{35}S]methionine to which orthovanadate was either added to 100 μM or omitted. Following incubation the cells were lysed and P130$^{gag-fps}$ was immunoprecipitated with an anti-p19^{gag} monoclonal antibody. P130$^{gag-fps}$ isolated from untreated cells migrated as a diffuse band during electrophoresis, with the radiolabel concentrated in the more rapidly moving form (Figure

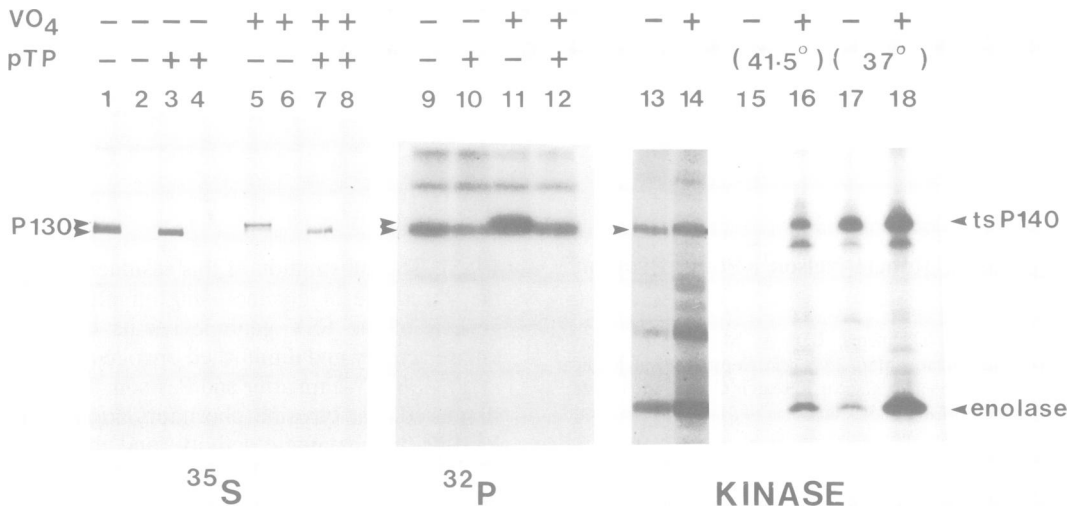

Fig. 1. Manipulation of P130$^{gag-fps}$ electrophoretic mobility, phosphorylation and kinase activity by orthovanadate and phosphotyrosyl phosphatase. FSV-transformed rat-2 cells were labeled for 14 h with [^{35}S]methionine (**lanes 1–8**) or for 4 h with $^{32}P_i$ (**lanes 9–12**). 100 μM sodium orthovanadate (**lanes 5–8,11,12**) or buffer alone (**lanes 1–4,9,10**) were added to the tissue culture medium for the final 4 h of radiolabeling. Cells were lysed and immunoprecipitated with R254E anti-p19^{gag} monoclonal antibody (**lanes 1,3,5,7,9–12**) or control antibody (**lanes 2,4,6,8**). After washing, immunoprecipitates were incubated with phosphotyrosyl phosphatase (pTPIII) (**lanes 3,4,7,8,10,12**) or buffer (**lanes 1,2,5,6,9,11**) for 10 min at 37°C. To measure the effect of orthovanadate pre-incubation on gap–fps kinase activity. FSV-transformed rat-2 cells (**lanes 13** and **14**) or *ts*FSV L5-infected CEFs (**lanes 15–18**) were incubated in the absence (**lanes 13,15,17**) or presence (**lanes 14,16,18**) of 100 μM orthovanadate for 4 h. *ts*FSV-infected CEFs were either maintained at 37°C throughout (**lanes 17,18**) or shifted from 37°C to 41.5°C at the start of the incubation period (**lanes 15,16**). Cells were lysed and immunoprecipitated with anti-p19^{gag} antibody, and the immunoprecipitates incubated with [γ-^{32}P]ATP and acid-denatured enolase. All samples were analyzed by SDS–PAGE and autoradiography.

1, lane 1). Treatment with 100 μM orthovanadate led to a pronounced shift in the distribution of P130$^{gag-fps}$ such that the majority of label was present in a more slowly migrating species (Figure 1, lane 5).

The possibility that these distinct forms of P130$^{gag-fps}$ resulted from differential phosphorylation was tested indirectly by using phosphotyrosyl phosphatase purified from chicken embryo fibroblasts. The ability of this enzyme preparation to strip phosphates from modified tyrosine residues was demonstrated using as a substrate for dephosphorylation P130$^{gag-fps}$ which had been specifically ^{32}P-labeled at tyrosine *in vitro* (see Figure 2). P130$^{gag-fps}$ immunoprecipitated from cells that had been metabolically labeled with [^{35}S]methionine in the presence or absence of 100 μM orthovanadate was therefore incubated with CEF phosphotyrosyl phosphatase. In each case phosphatase activity converted the more slowly migrating forms of P130$^{gag-fps}$ to a rapidly migrating band (Figure 1; compare lanes 5 and 7). The interconversion of the different [^{35}S]methionine-labeled P130$^{gag-fps}$ species by the *in vitro* addition or intracellular inhibition of phosphotyrosyl phosphatase indicated that they indeed represent distinct phosphorylation states of the protein. Phosphorylation of P130$^{gag-fps}$ was measured directly by metabolic labeling of FSV-transformed rat-2 cells with $^{32}P_i$ for 4 h. ^{32}P-labeled P130$^{gag-fps}$ isolated from cells that had been incubated concurrently with ^{32}P and 100 μM orthovanadate migrated more slowly and contained more phosphate per mole of protein than did P130$^{gag-fps}$ labeled in the absence of orthovanadate (Figure 1). Phosphoamino acid analysis showed that the slowly migrating form of P130$^{gag-fps}$ from orthovanadate-treated cells contained 4-fold more phosphotyrosine with respect to phosphoserine than P130$^{gag-fps}$ from cells radiolabeled in the absence of phosphatase inhibitor (Figure 3). Therefore the higher mol. wt forms of P130$^{gag-fps}$ which are particularly abundant following orthovanadate treatment result from increased stoichiometry of tyrosine phosphorylation.

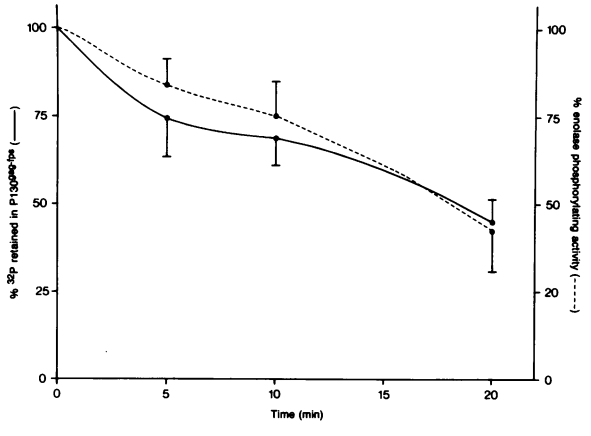

Fig. 2. Effect of phosphotyrosyl phosphatase treatment on P130$^{gag-fps}$ kinase activity. Immunoprecipitated P130$^{gag-fps}$ specifically ^{32}P-labeled at tyrosine by *in vitro* autophosphorylation was incubated for increasing lengths of time with CEF phosphotyrosyl phosphatase, and the extent of dephosphorylation at tyrosine was inferred from the ^{32}P c.p.m. remaining in P130$^{gag-fps}$. Partially dephosphorylated P130$^{gag-fps}$ was then introduced into a brief second kinase reaction in which enolase served as an exogenous substrate for tyrosine phosphorylation. The percentage of ^{32}P remaining in P130$^{gag-fps}$ (—) and the extent of enolase phosphorylation subsequently induced by the same sample, i.e. % remaining kinase activity (----), are plotted versus time of incubation with phosphotyrosyl phosphatase. Each data point is normalized to a control treated identically except for the omission of phosphatase. Each point corresponds to an average of four determinations with error bars representing the standard error of the mean.

To identify the sites that participate in this enhanced phosphorylation, two-dimensional tryptic phosphopeptide maps were prepared from ^{32}P-labeled P130$^{gag-fps}$ isolated from orthovanadate-treated or untreated cells (Figure 4). In the absence of orthovanadate P130$^{gag-fps}$ is phosphorylated on at least three tyrosine residues including Tyr 1073 (Figure 4, spots 3a–c) and more N-terminal sites (spots 4 and 7), in addition to serine resi-

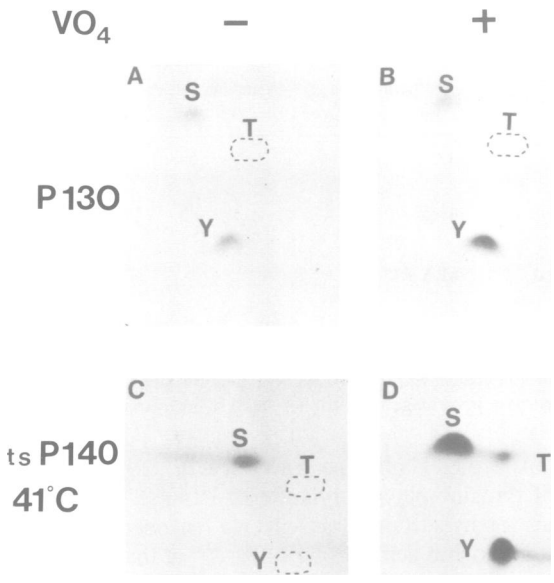

Fig. 3. Phosphoamino acid analysis of *gag−fps* proteins isolated from orthovanadate-treated cells. FSV-transformed rat-2 cells **(panels A** and **B)** and *ts*FSV-infected CEFs at 41.5°C **(panels C** and **D)** were metabolically labeled with $^{32}P_i$ for 4 h in the absence **(panels A** and **C)** or presence **(panels B** and **D)** of 100 μM sodium orthovanadate. ^{32}P-labeled P130$^{gag-fps}$ and *ts*P140$^{gag-fps}$ were isolated by immunoprecipitation and gel electrophoresis, and subjected to acid hydrolysis. Phosphoamino acids were separated by two-dimensional electrophoresis, and the plates exposed to X-ray film for 14 days with an intensifying screen. Marker phosphoamino acids (phosphoserine-S, phosphothreonine-T, phosphotyrosine-Y) were identified with ninhydrin.

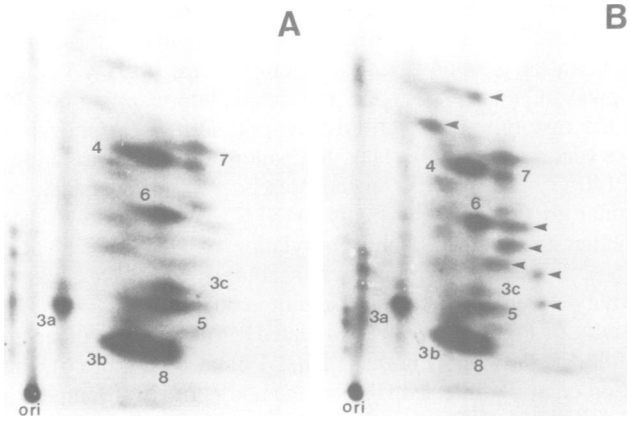

Fig. 4. Tryptic phosphopeptides of P130$^{gag-fps}$ from orthovanadate-treated or untreated cells. FSV-transformed rat-2 cells were labeled for 4 h with $^{32}P_i$ in the absence **(panel A)** or presence **(panel B)** of 100 μM sodium orthovanadate. ^{32}P-labeled P130$^{gag-fps}$ was recovered by immunoprecipitation and gel electrophoresis, and digested with TPCK-trypsin. Tryptic phosphopeptides were separated in two dimensions by electrophoresis at pH 2.1 (first dimension; anode to the left, cathode to the right) followed by chromatography (second dimension, from bottom to top). Spots are numbered as in Weinmaster *et al.* (1984). See text for further details.

dues contained in peptides 5, 6 and 8. The maps were qualitatively similar indicating that increased tyrosine phosphorylation of P130$^{gag-fps}$ in the presence of orthovanadate might be largely accounted for by enhanced stoichiometry of phosphorylation at previously identified sites. There was, however, increased phosphorylation of some previously very minor peptides (arrowed

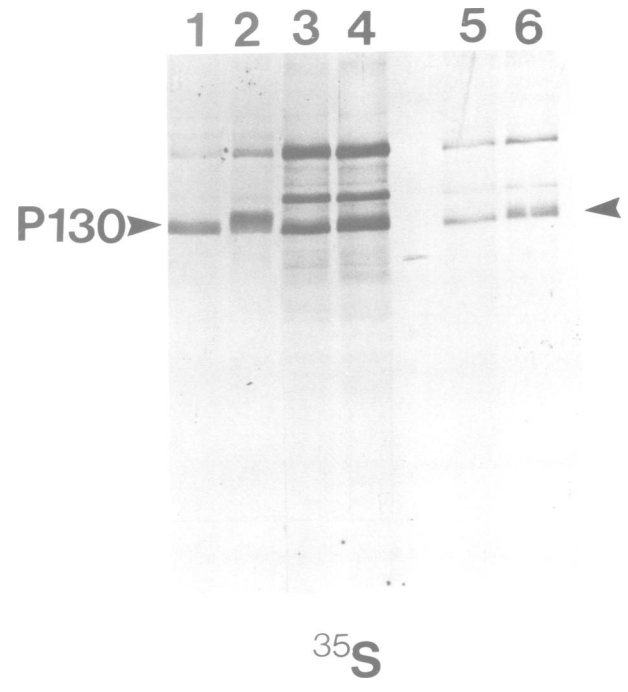

Fig. 5. Tyr 1073 is required for hyperphosphorylation of P130$^{gag-fps}$. Rat-2 cells transformed with *wt*FSV **(lanes 1** and **2)** or with FSV mutants encoding P130$^{gag-fps}$ proteins with serine **(lanes 3** and **4)** or phenylalanine **(lanes 5** and **6)** at residue 1073 were labeled with [^{35}S]methionine for 4 h in the absence **(lanes 1, 3** and **5)** or presence **(lanes 2, 4** and **6)** of 100 μM sodium orthovanadate. Cells were lysed and immunoprecipitated with anti-p19^{gag} antibody. Immunoprecipitates were analyzed by SDS−PAGE and autoradiography.

in Figure 4). To probe whether Tyr 1073 was important in the response to orthovanadate we used two FSV mutants, FSV-F(1073) and FSV-S(1073), in which the codon for Tyr 1073 of P130$^{gag-fps}$ is altered to those for phenylalanine or serine, thereby blocking autophosphorylation at this residue (Weinmaster *et al.*, 1984; Weinmaster and Pawson, 1986). Incubation of [^{35}S]-methionine-labeled rat-2 cells expressing the mutant P130$^{gag-fps}$ proteins with orthovanadate led to a detectable decrease in P130$^{gag-fps}$ mobility, but the apparent shift in mol. wt was small when compared with that seen for wild-type P130$^{gag-fps}$ (Figure 5). These results indicate that phosphorylation of Tyr 1073 is a principal requirement for the mobility shift induced by orthovanadate, but that other tyrosine sites may also contribute to this response independently of Tyr 1073.

Increased tyrosine phosphorylation of P130$^{gag-fps}$ correlates with increased in vitro protein-tyrosine kinase activity

The inclusion of 100 μM orthovanadate in all buffers from the point of cell lysis produced a small decrease in P130$^{gag-fps}$ mobility and a modest increase in kinase activity measured using enolase as an exogenous substrate for phosphorylation. A more pronounced (3- to 5-fold) elevation in activity was observed when P130$^{gag-fps}$ was isolated from transformed rat-2 cells that had been pre-incubated with 100 μM orthovanadate (Figure 1, lanes 13 and 14). Initial *in vitro* P130$^{gag-fps}$ reaction rates at ATP concentrations up to 500 μm were increased by orthovanadate pre-incubation (data not shown). No detectable increase in kinase activity was observed when similar experiments were undertaken with cells expressing FSV-S(1073) or FSV-F(1073) P130$^{gag-fps}$ (Figure 6), suggesting that phosphorylation of Tyr 1073 was important to the orthovanadate-induced stimulation of P130$^{gag-fps}$ kinase activity.

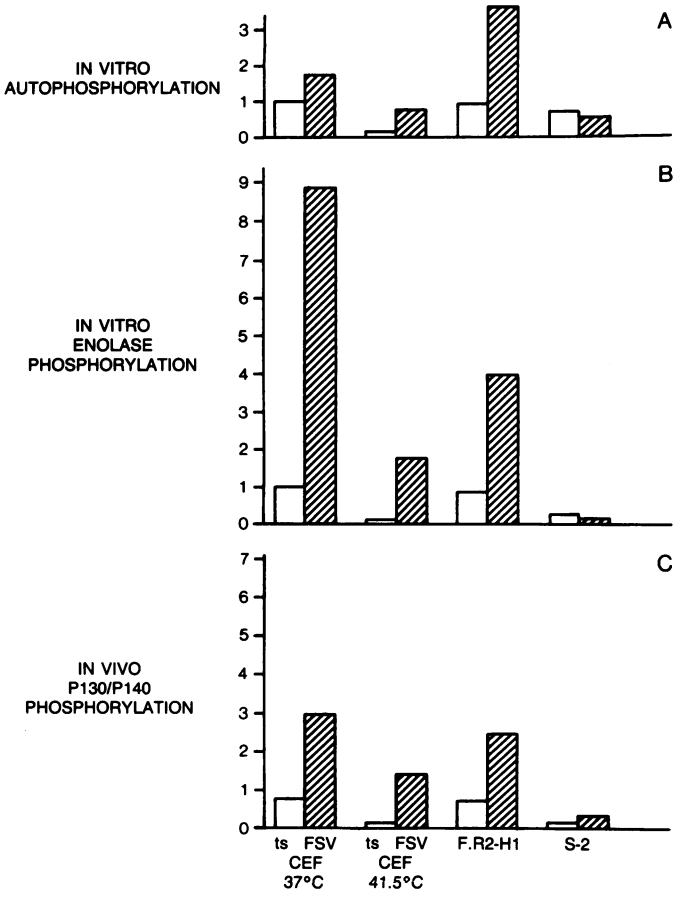

Fig. 6. Quantitation of phosphorylation and kinase activity of *gag-fps* proteins. *ts*FSV-infected CEFs (at 37°C or 41.5°C) or rat-2 cells expressing *wt*FSV or FSV-S(1073) P130$^{gag-fps}$ (S-2) were analyzed for *in vivo* phosphorylation and *in vitro* kinase activity of *gag-fps* proteins. Cells were either untreated (open bars), or pretreated with 100 μM sodium orthovanadate for 4 h (shaded bars). All values were normalized to those obtained for *wt*P130$^{gag-fps}$ or *ts*P140$^{gag-fps}$ at 37°C from untreated cells. **Panel A:** P130 or P140$^{gag-fps}$ *in vitro* autophosphorylation. **Panel B:** *In vitro* enolase phosphorylation. **Panel C:** *In vivo* phosphorylation of *gag-fps* proteins.

In the reciprocal experiment P130$^{gag-fps}$ was immunoprecipitated from FSV-transformed cells, briefly allowed to autophosphorylate using [γ-^{32}P]ATP, and then incubated with phosphotyrosyl phosphatase. Dephosphorylation of P130$^{gag-fps}$ tyrosine was monitored by measuring the loss of ^{32}P label during the phosphatase reaction. Partially dephosphorylated P130$^{gag-fps}$ was then introduced into a brief second kinase reaction containing [γ-^{32}P]ATP and enolase. Relative to control reactions a 2-fold decrease in P130$^{gag-fps}$ phosphorylation resulted in an ~2-fold decrease in enolase phosphorylation (Figure 2).

tsFSV P140$^{gag-fps}$ phosphorylation and kinase activity at the non-permissive temperature for transformation are restored by orthovanadate

A variant of the original FSV isolate (*ts*FSV L5) encodes a 140-kd transforming protein which is temperature-sensitive for transformation (Pawson *et al.*, 1980; Lee *et al.*, 1981). At the non-permissive temperature P140$^{gag-fps}$ of *ts*FSV L5 rapidly becomes dephosphorylated at tyrosine and serine, loses ~95% of protein-tyrosine kinase activity and dissociates from the particulate fraction of the cytoplasm, events that are associated with loss

Table I. Incorporation of ^{32}P into whole cell phosphotyrosine in response to orthovanadate

	Temperature (°C)	Vanadate[b]	Whole cell phosphotyrosine[c]
*ts*FSV	37	−	0.4
		+	14.3
CEF	41.5[a]	−	0.1
		+	14.0
Uninfected	41.5	−	0.05
CEF		+	7.0

[a]Cells were shifted to 41.5°C 24 h prior to addition of orthovanadate.
[b]Cells were incubated with (+) or without (−) 100 μM orthovanadate.
[c]Expressed as a percentage of phosphorylated hydroxyamino acids.

of the transformed phenotype (Pawson *et al.*, 1980; Moss *et al.*, 1984). If tyrosine phosphorylation of P140$^{gag-fps}$ were in fact important for its sustained activity we reasoned that we might resurrect the kinase activity of P140$^{gag-fps}$ at the non-permissive temperature in *ts*FSV-infected chicken embryo fibroblasts (CEFs) by incubating these cells with orthovanadate.

As previously noted P140$^{gag-fps}$ from *ts*FSV-infected CEFs maintained at 41.5°C for 4 h was poorly phosphorylated in comparison with P140$^{gag-fps}$ from cells maintained at 37°C (Figure 6). Incubation with 100 μM orthovanadate for 4 h led to enhanced phosphorylation of P140$^{gag-fps}$ at 37°C and a dramatic 30- to 100-fold increase in P140$^{gag-fps}$ phosphorylation at the non-permissive temperature (Figure 6). The phosphorylation of P140$^{gag-fps}$ could be restored even when the cells had been maintained at 41.5°C for 24 h prior to the addition of orthovanadate (data not shown). Phosphoamino acid analysis of P140$^{gag-fps}$ showed the reappearance of phosphotyrosine and a substantial increase in phosphoserine following orthovanadate treatment at the non-permissive temperature (41.5°C) (Figure 3, panels C and D). Tryptic phosphopeptide mapping confirmed the restoration of both tyrosine and serine phosphorylation sites (data not shown). Thus the pattern of phosphorylation of *ts*P140$^{gag-fps}$ at 41.5°C in the presence of orthovanadate was similar to that seen normally at 37°C. We wished to know whether the increased phosphorylation of *ts*P140$^{gag-fps}$ at the non-permissive temperature was accompanied by altered kinase activity. Even at the permissive temperature, the *in vitro* kinase activity of P140$^{gag-fps}$ was stimulated 10-fold by pre-incubation with orthovanadate (Figure 1, lanes 17 and 18; Figure 6). Incubation of *ts*FSV-infected CEFs at the non-permissive temperature (41.5°C) with 100 μm orthovanadate stimulated *in vitro* phosphorylation of enolase by P140$^{gag-fps}$ by some 40-fold (Figure 1, lanes 15 and 16). The *in vitro* kinase activity of P140$^{gag-fps}$ from orthovanadate-treated cells at 41.5°C exceeded the activity of P140$^{gag-fps}$ from untreated *ts*FSV-transformed cells at 37°C (Figure 6). The stimulation of P140$^{gag-fps}$ kinase activity from *ts*FSV-infected CEFs at the non-permissive temperature required that the cells be pre-incubated with orthovanadate, and was lost if orthovanadate was omitted from the cell lysis buffer (data not shown). These results indicate that the increased phosphorylation induced by orthovanadate at 41.5°C is associated with a greatly increased P140$^{gag-fps}$ kinase activity.

Phenotypic effects of orthovanadate-induced reactivation of tsFSV P140$^{gag-fps}$

To confirm that orthovanadate was blocking phosphotyrosyl phosphatase activity in CEFs, we examined whole cell phosphoamino acids in uninfected and *ts*FSV-infected CEFs at 41.5°C in the

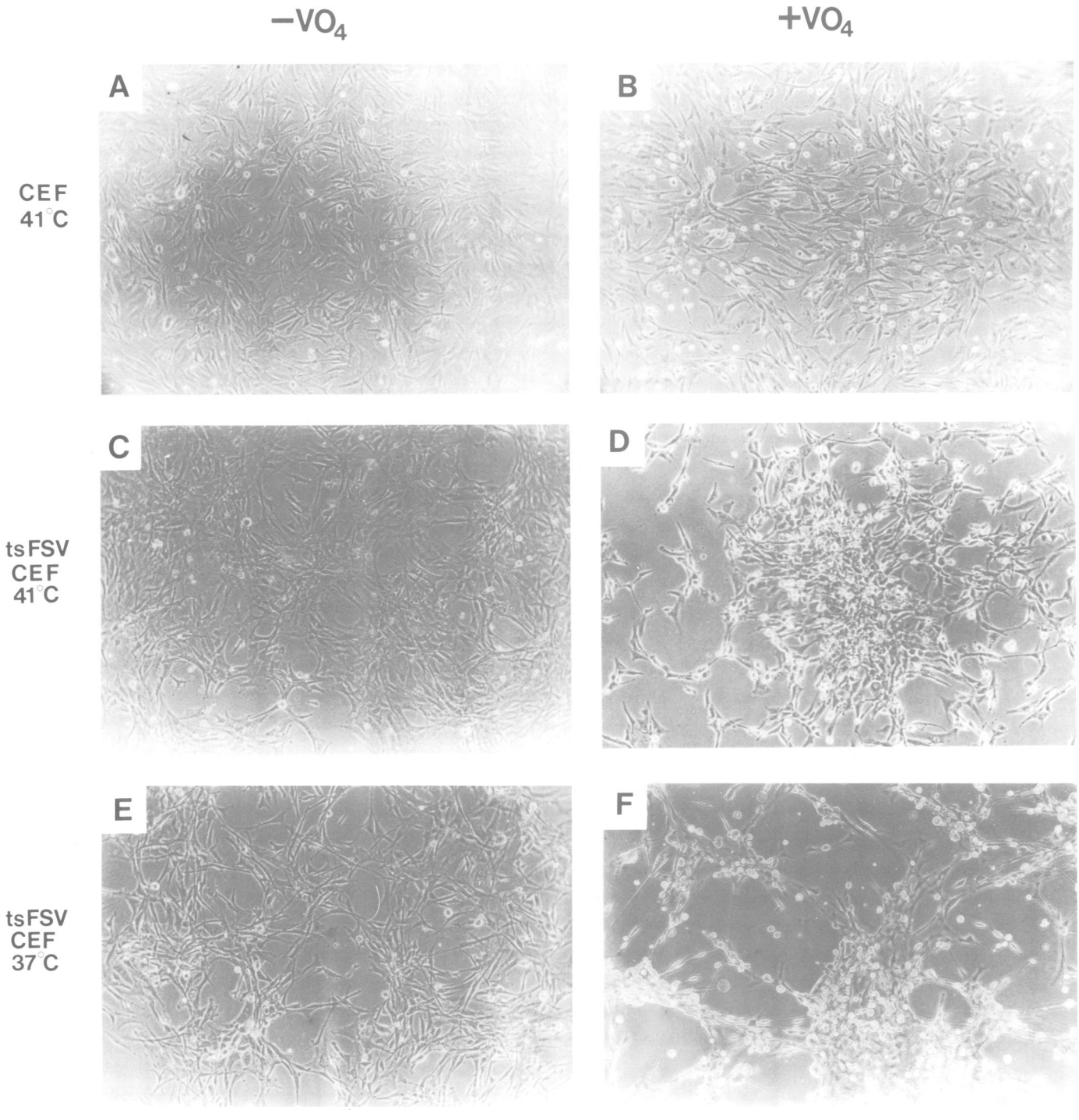

Fig. 7. Morphology of chicken embryo fibroblasts. Phase contrast photomicrographs of untreated cells (**panels A, C and E**) or duplicate plates incubated with 100 μM orthovanadate for 4 h (**panels B, D and F**). **Panels A** and **B:** uninfected CEFs at 41.5°C; **panels C** and **D:** *ts*FSV-infected CEFs maintained at 41.5°C for 20 h prior to and during orthovanadate treatment; **panels E** and **F:** *ts*FSV-infected CEFs grown at 37°C throughout.

presence and absence of orthovanadate. A 4-h incubation with 100 μM orthovanadate induced a striking increase in whole cell phosphotyrosine from 0.05% to 7% of total phosphoamino acids in uninfected cells and to 14% in *ts*FSV-infected cells (Table I). These achieved levels of whole cell phosphotyrosine following orthovanadate treatment are rather remarkable and support the idea that inhibition of CEF phosphotyrosyl phosphatases is a major activity of orthovanadate. The additional 7% of phosphotyrosine seen in treated, infected cells (both at 37°C and 41.5°C) compared with uninfected CEFs is presumably due to the presence of the stimulated $P140^{gag-fps}$.

To establish whether the reactivation of *ts*FSV $P140^{gag-fps}$ kinase activity at 41.5°C had an effect on the cell phenotype, we examined the morphology of CEFs (both infected and uninfected) treated with 100 μM orthovanadate (Figure 7). Since prolonged orthovanadate treatment was cytotoxic an incubation period of 4 h was used, and cell morphology examined by phase contrast microscopy. *ts*FSV-Infected cells at both permissive

and non-permissive temperatures showed a marked progression towards a highly rounded refractile morphology during a 4-h incubation with orthovanadate, compared with a much less obvious transition by uninfected CEFs. Thus infected cells at 41.5°C went from a relatively flat morphology similar to uninfected CEFs, to give more rounded and spindle-shaped cells upon orthovanadate treatment. Even infected cells maintained at 37°C which had already transformed became much more refractile and rounded when incubated with orthovanadate.

We conclude that at the non-permissive temperature for transformation, phosphotyrosyl phosphatase inhibition is accompanied by a large increase in phosphorylation of *ts*FSV P140$^{gag-fps}$, restoration of P140$^{gag-fps}$ kinase activity and the rapid reappearance of a transformed cell morphology.

Discussion

Autophosphorylation at tyrosine stimulates P130$^{gag-fps}$ kinase activity

Incubation of FSV-transformed cells with orthovanadate converted the majority of P130$^{gag-fps}$ to a slowly migrating species that contained increased phosphotyrosine. This effect was to some extent reversible by the incubation of P130$^{gag-fps}$ with purified chicken phosphotyrosyl phosphatase. P130$^{gag-fps}$ contains at least three tyrosine autophosphorylation sites (see Figure 4) (Weinmaster *et al.*, 1984). Partial phosphorylation of these tyrosine residues might account for the multiple P130$^{gag-fps}$ species normally identified by gel electrophoresis. Analysis of P130$^{gag-fps}$ *in vitro* kinase activity showed that hyperphosphorylation of P130$^{gag-fps}$ resulted in increased kinase activity which was dependent on the presence of Tyr 1073, whereas activity was lowered following partial tyrosine dephosphorylation of *wt*P130$^{gag-fps}$ with phosphotyrosyl phosphatase. These observations substantiate the suggestion that phosphorylation at Tyr 1073 augments the enzymatic function of P130$^{gag-fps}$. The stimulation of P130$^{gag-fps}$ activity by orthovanadate is apparently a consequence of phosphatase inhibition rather than a direct effect on P130$^{gag-fps}$, for example by spontaneous esterification of tyrosine by orthovanadate (Tracey and Gresser, 1986), as orthovanadate added to P130$^{gag-fps}$ *in vitro* induced only a minor increase in kinase activity. Furthermore, orthovanadate had no effect on the kinase activity of functional v-*fps* polypeptides expressed in *Escherichia coli* even when the bacteria were grown in the presence of orthovanadate (I.Sadowski and T.Pawson, unpublished results). Since bacteria lack detectable phosphotyrosyl phosphatases, this observation supports the contention that orthovanadate affects P130$^{gag-fps}$ kinase activity in eucaryotic cells indirectly by phosphatase incubation.

Phenotypic suppression of tsFSV defect by phosphotyrosyl phosphatase inhibition

We have found that the enzymatic and biological defect of P140$^{gag-fps}$ encoded by a *ts*FSV variant is overcome by incubating infected cells held at the non-permissive temperature for transformation with orthovanadate. The simplest explanation of these results is that at the non-permissive temperature the phosphotyrosine residues of *ts*P140$^{gag-fps}$ have increased sensitivity or accessibility to phosphotyrosyl phosphatases; orthovanadate, by inhibiting phosphatase activity, may allow the accumulation of phosphotyrosine on P140$^{gag-fps}$ and thereby prevent or reverse its conversion to an inactive dephosphorylated form. Consistent with this suggestion, stimulation of P140$^{gag-fps}$ tyrosine kinase activity by orthovanadate at 41.5°C is again reversed if the phosphatase inhibitor is omitted from the cell lysis buffer, indicating that at the elevated temperature the *ts*FSV L5 protein is highly sensitive to phosphatase action. These results suggest that tyrosine phosphorylation is a potent regulator of P140$^{gag-fps}$ function, and that the loss of phosphate from tyrosine at 41.5°C may normally initiate the drop in *ts*P140$^{gag-fps}$ kinase activity and elution of the protein from the particulate to the soluble phase of the cytoplasm. The general significance of phosphotyrosyl phosphatases in regulating protein-tyrosine kinase action is evident from the remarkable increase in CEF phosphotyrosine content induced by 100 μM orthovanadate even in uninfected cells.

A conserved tyrosine residue is a site for positive regulation of protein-tyrosine kinases

Tyrosine phosphorylation of cytoplasmic protein-tyrosine kinases has been reported to have diverse effects, but two general themes are becoming apparent. Autophosphorylation at a conserved tyrosine within the catalytic domain (Tyr 1073 of P130$^{gag-fps}$) appears to stimulate kinase activity. The presence of this tyrosine residue in all protein-tyrosine kinases suggests a critical function, although unlike other conserved sequences it is clearly not essential for catalysis in either P130$^{gag-fps}$ or p60$^{v\text{-}src}$ (Snyder *et al.*, 1983; Cross and Hanafusa, 1983; Weinmaster *et al.*, 1984; Weinmaster and Pawson, 1986). Preliminary data suggest that regulation of enzymatic function by phosphorylation of this residue may be a more general phenomenon among tyrosine kinases. Autophosphorylation of either of two adjacent tyrosines at the corresponding site in the insulin-receptor subunit is apparently required for the insulin-dependent activation of receptor kinase activity and hexose uptake (Rosen *et al.*, 1983; Ellis *et al.*, 1986). Phosphorylation at the homologous Tyr 416 of p60$^{c\text{-}src}$ may also be necessary for stimulation of c-*src* kinase activity and transforming ability (T.Kmiecik and D.Shalloway, personal communication). In contrast it is proposed that phosphorylation of p60$^{c\text{-}src}$ with a C-terminal non-catalytic domain at Tyr 527, presumably by a cellular kinase, may repress kinase activity (Courtneidge, 1985; Cooper *et al.*, 1986; Cartwright *et al.*, 1986). This putative negative regulatory sequence is shared between a number of cellular cytoplasmic protein-tyrosine kinases (Cooper *et al.*, 1986; Cartwright *et al.*, 1986), but is absent from c-*fps/fes* gene products (Huang *et al.*, 1985; Roebroek *et al.*, 1985). Therefore tyrosine phosphorylation at different sites may have either a positive or an inhibitory effect on the activity of protein-tyrosine kinases.

Tyrosine autophosphorylation as a molecular switch in signal transmission

fps/fes-encoded cytoplasmic proteins are apparently present in the cell as relatively inactive, poorly phosphorylated soluble polypeptides or as active membrane-associated phosphoproteins (Adkins and Hunter, 1982; Adkins *et al.*, 1982; Feldman *et al.*, 1983; Moss *et al.*, 1984; Young and Martin, 1984). The data presented here suggest that tyrosine phosphorylation or dephosphorylation may serve as a trigger for the interconversion of these species.

The normal c-*fps/fes* product is specifically expressed in hematopoietic cells of the myeloid lineage (Feldman *et al.*, 1985; MacDonald *et al.*, 1985; Samarut *et al.*, 1985), and has been implicated in the response to colony stimulating factors (Feldman *et al.*, 1985; Carmier and Samarut, 1986). We speculate that autophosphorylation of c-*fps/fes* proteins induces a transient stimulation of their kinase activity during myelopoiesis. The extent and duration of activation in such a system would depend on the interplay between tyrosine kinase and phosphatase activities, and would not necessarily require the continued presence of the original stimulus. In this sense tyrosine autophosphorylation may

normally serve as a switch by which tyrosine kinases are activated by extracellular stimuli, and by which these signals are prolonged. There are several precedents for this idea including that afforded by the insulin receptor (Ellis *et al.*, 1986). Recent theoretical and experimental data have implicated autophosphorylation by neural protein kinases as a mechanism by which stimulatory signals might be extended (Lisman, 1985; Miller and Kennedy, 1986). In particular autophosphorylation of the type II Ca^{2+}/calmodulin-dependent protein kinase from brain can abrogate its dependency on Ca^{2+}/calmodulin for kinase activity (Miller and Kennedy, 1986). In these examples caution should perhaps be applied in extrapolating from *in vitro* kinase experiments undertaken with sub-physiological ATP concentrations.

The role of cytoplasmic tyrosine kinases such as c-*fps/fes* proteins might normally be to serve as substrates for transmembrane kinase or as catalytic subunits of transmembrane receptors lacking effector domains. The FSV-transforming protein apparently differs from its normal counterpart in undergoing constitutive autophosphorylation, and this may partly explain its oncogenic activity.

Materials and methods

Cells and viruses

Rat-2 cells (Topp, 1981) transfected with wild-type or mutant FSV DNAs, were isolated as foci, and cloned by growth in soft agar (Weinmaster *et al.*, 1984; Weinmaster and Pawson, 1986). The F.R2-H1 cell line expresses *wt*FSV $P130^{gag-fps}$, S-2 expresses FSV-S(1073) $P130^{gag-fps}$ with a Tyr→Ser substitution at residue 1073, and F3 expresses FSV-F(1073) $P130^{gag-fps}$ with a Tyr→Phe change at residue 1073. Rat cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C. Chicken embryo fibroblasts were cultured in DMEM containing 2% tryptose phosphate broth, 1% calf serum, 1% chicken serum. CEFs were infected with the *ts*FSV L5 virus strain pseudotyped with Fujinami associated virus (FAV) and maintained at 37°C or 41.5°C as described previously (Pawson *et al.*, 1980). For incubation of cells with orthovanadate, a 1 mM solution of Na_3VO_4 in 2 mM Tris−HCl pH 8.2 was pre-warmed to the appropriate temperature and added to the tissue culture medium, usually to give a final concentration of 100 μM. Control plates received an equal volume of 2 mM Tris−HCl pH 8.2.

Metabolic radiolabeling and immunoprecipitation

Cells were radiolabeled with [^{35}S]methionine (Amersham; 1000 Ci/mmol) at 100 μCi/ml in DMEM lacking non-radioactive methionine and containing 2% FBS, or with $^{32}P_i$ (ICN; carrier-free) at 1 mCi/ml in DMEM lacking phosphate and containing 2% FBS. Most labeling periods were 4 h. [^{35}S]Methionine-labeled cells were lysed in RIPA buffer (10 mM Tris−HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate (SDS), 200 μg/ml leupeptin, 100 μM Na_3VO_4). ^{32}P-labeled cells were lysed in kinase lysis buffer (20 mM Tris−HCl pH 7.5, 150 mM NaCl, 1mM EDTA, 1% w/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 200 μg/ml leupeptin and unless otherwise indicated 100 μM Na_3VO_4). Cell lysates were clarified and immunoprecipitated with a mouse anti-$p19^{gag}$ monoclonal antibody (R254E) as described (Weinmaster *et al.*, 1983, 1984). Washed immune complexes were analyzed by electrophoresis through 7.5% SDS−polyacrylamide gels, followed by exposure of the dried gels to X-ray film (XAR-5, Kodak). Radioactivity in specific *gag−fps* proteins was assessed by excision of gel bands followed by liquid scintillation counting, and was normalized to the total incorporation of ^{32}P or ^{35}S into trichloroacetic acid (TCA)-precipitable protein.

Immune complex kinase reaction

Samples of $10^6-5 \times 10^7$ cells were washed and lysed in kinase lysis buffer and immunoprecipitated as described previously (Weinmaster *et al.*, 1984). Immune complexes were incubated in 50-μl reaction mixtures containing 10 mM $MnCl_2$, 20 mM Hepes pH 7.4, 5−30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 100 μM Na_3VO_4 unless otherwise indicated for 15 min at 22°C. Acid-denatured rabbit muscle enolase was included as an exogenous substrate at 0.1 mg/ml as described previously (Cooper *et al.*, 1984; Weinmaster *et al.*, 1984). The *in vitro* incorporation of ^{32}P into *gag-fps* proteins or enolase was normalized to the amount of *gag-fps* protein in the immunoprecipitate determined by [^{35}S]methionine-labeling and immunoprecipitation of sibling cultures.

Tryptic phosphopeptide analysis

^{32}P-labeled proteins were eluted from gel slices, TCA-precipitated, oxidized with performic acid and digested wtth tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin as described earlier (Pawson *et al.*, 1980; Weinmaster *et al.*, 1983). Tryptic digests were separated by electrophoresis at 1 kV for 60 min at pH 2.1 on t.l.c. plates (thickness 0.1 mm; E.Merck AG), followed by chromaography in *n*-butanol−acetic acid−water−pyridine (75:15:60:50) in the second dimension. Dried plates were exposed to X-ray film in the presence of an intensiying screen for 5−14 days at −80°C.

Phosphoamino acid analysis

^{32}P-labeled proteins were eluted from gel slices, TCA-precipitated and subjected to acid hydrolysis in 6 N HCl at 110°C for 60 min as detailed elsewhere (Cooper *et al.*, 1983). For whole cell phosphoamino acids, proteins were extracted from lysates of cells labeled with $^{32}P_i$ as previously described (Cooper *et al.*, 1983). The acid hydrolysates were subjected to electrophoresis at pH 1.9 in the first dimension at 1 kV for 150 min and at pH 3.5 in the second dimension for 80 min. Plates were fluorographed as for tryptic phosphopetide maps. The identities of radioactive spots were determined by co-migration with ninhydrin-stained phosphoamino acid markers. Radioactivity in phosphoamino acid spots was estimated by scraping the appropriate spots and liquid scintillation counting.

Phosphotyrosyl phosphatase purification

The phosphotyrosyl phosphatase pTPIII was partially purified from CEFs by anion exchange chromatography, as previously described (Nelson and Branton, 1983).

Phosphatase assays

Immune complexes from radiolabeled cells, or from immune complex kinase reactions, were prepared as described above. Immune complexes were then washed twice in phosphatase buffer (20 mM Tris−HCl pH 7.0, 250 mM sucrose, 20 mM NaCl, 4 mM β-mercaptoethanol, 2 mM dithiothreitol, 50 mM sodium fluoride, 1 mM EDTA and 200 μg/ml leupeptin). Washed immune precipitates were suspended in 40 μl phosphatase buffer with or without partially purified chicken embryo fibroblast phosphotyrosyl phosphatase and incubated at 37°C for varying lengths of time. Reactions were severed by immersion in liquid nitrogen. Where samples were to be retested for kinase activity following phosphatase treatment, kinase lysis buffer including orthovanadate was added to stop phosphatase reactions. Phosphatased immunoprecipitates were washed several times in kinase lysis buffer followed by several washes in kinase reaction buffer. Finally immune precipitates were suspended in kinase reaction buffer containing acid-denatured enolase and 30 μCi [γ-^{32}P]ATP and incubated at 30°C for 2 min. Reactions were stopped with the addition of an equal volume of 2 × SDS sample buffer, and immune complex proteins analyzed by SDS−PAGE, followed by autoradiography.

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