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Identification and characterization of p59^{fyn} (a *src*-like protein tyrosine kinase) in normal and polyoma virus transformed cells

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***fyn* is a member of the growing family of protein tyrosine kinase genes whose sequences are highly related to that of c-*src*. We have generated antibodies to peptides corresponding to two different amino-terminal sequences encoded by this gene. Antisera to both peptides recognized a 59 kd protein from human and mouse fibroblasts. p59^{fyn} was phosphorylated *in vivo* on serine and tyrosine residues and was also myristylated. Furthermore, immune precipitates of p59^{fyn} had tyrosine kinase activity *in vitro*, as measured by autophosphorylation and by phosphorylation of substrates such as enolase. This kinase activity was shown to be negatively regulated by tyrosine phosphorylation. We have also established that, like pp60$^{c\text{-}src}$ and p62$^{c\text{-}yes}$, p59^{fyn} was complexed with middle T antigen, the transforming protein of polyoma virus. However, the tyrosine kinase activity of p59^{fyn} was not elevated in middle T transformed cells. Possible explanations for this are discussed.**

Key words: middle T antigen/p59^{fyn}/protein tyrosine kinase/*src* family

Introduction

Most protein tyrosine kinases can be placed into one of two distinct classes (reviewed in Hunter and Cooper, 1985, 1986). Members of the first class are integral membrane proteins, of which some are receptors. This class includes the insulin and epidermal growth factor receptors. The second class contains membrane associated kinases which lack a transmembrane domain. The prototype of the latter class is pp60$^{c\text{-}src}$, the cellular homologue of the Rous sarcoma virus transforming protein pp60$^{v\text{-}src}$. Some protein tyrosine kinases (e.g. the products of the *fps* and *abl* genes), although not so easily classified, are nevertheless highly related to the *src* family.

The genes encoding many of the second class of tyrosine kinase have recently been cloned, and at the present time the *src*-like family has seven members (*src, fgr, yes, fyn, lyn, lck, hck*). Sequence comparison has revealed that the genes are homologous not only in their catalytic domains (which comprises ~40% of the protein) but also throughout much of the rest of the molecule (for a review see Cooper, 1988). The putative protein products of these genes therefore share a number of important sequence motifs. Firstly, they begin with the dipeptide Met-Gly; in pp60$^{c\text{-}src}$ the amino-terminal methionine is cleaved and the glycine is myristylated (Buss and Sefton, 1985). Myristylation has been shown to be necessary for membrane association (Cross *et al.*, 1984). Secondly, all the genes encode a sequence at the carboxyl terminus which contains a tyrosine. In pp60$^{c\text{-}src}$ the phosphorylation state of this residue (Tyr-527) plays a major role in the regulation of kinase activity (Courtneidge, 1985; Cooper *et al.*, 1986; Laudano and Buchanan, 1986). Thirdly, there is another domain which is common among all members of the *src* family and the proteins encoded by the *abl* and *fps* genes. This region, which has been called SH2, consists of ~100 residues amino-terminal to the catalytic domain. Mutations in the SH2 region of p130$^{gag\text{-}fps}$ and pp60$^{v\text{-}src}$ impair transforming ability (Bryant and Parsons, 1982; Kitamura and Yoshida, 1983; Cross *et al.*, 1985; Sadowski *et al.*, 1986). This same region contains two of the three domains recently shown to have homology to the gene for phospholipase C (Mayer *et al.*, 1988) and the *crk* oncogene (Stahl *et al.*, 1988). The major sequence divergence among *src* family members lies in the sequences coding for ~80 amino acids after Gly-2. The function of this region in pp60$^{c\text{-}src}$ is not clearly defined, although it contains two serine phosphorylation sites; one phosphorylated by the cAMP-dependent protein kinase (Collett *et al.*, 1979; Roth *et al.*, 1983) and one by protein kinase C (Gould *et al.*, 1985; Gentry *et al.*, 1986). The mutations which have been created in this region of pp60$^{v\text{-}src}$ do not appear to abolish kinase activity or transformation; rather, cells expressing these mutant proteins often display fusiform morphology and a partially transformed phenotype (Cross and Hanafusa, 1983). It is possible that both this region and the SH2 region function in, for example, interaction with other proteins and/or regulatory molecules.

Three members of the *src* family (*src, yes* and *fgr*) have viral homologues that cause malignant transformation (see Hunter and Cooper, 1986). The viral oncogenes differ from their normal cellular counterparts by several mutations, but in all cases the alterations include the replacement of the tyrosine homologous Tyr-527 in pp60$^{c\text{-}src}$. Furthermore, pp60$^{c\text{-}src}$ in which Tyr-527 is mutated to Phe transforms cells (Cartwright *et al.*, 1987; Kmiecik *et al.*, 1987; Piwnica-Worms *et al.*, 1987) and the oncogenic potential of another *src* family member, *lck*, has been unmasked by the corresponding mutation (Amrein and Sefton, 1988; Marth *et al.*, 1988). These results highlight the importance of the carboxy terminus in regulating activity and suggest that similar manipulations may render the other members of the family oncogenic.

Both pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983) and pp62$^{c\text{-}yes}$ (Kornbluth *et al.*, 1987) are found complexed to

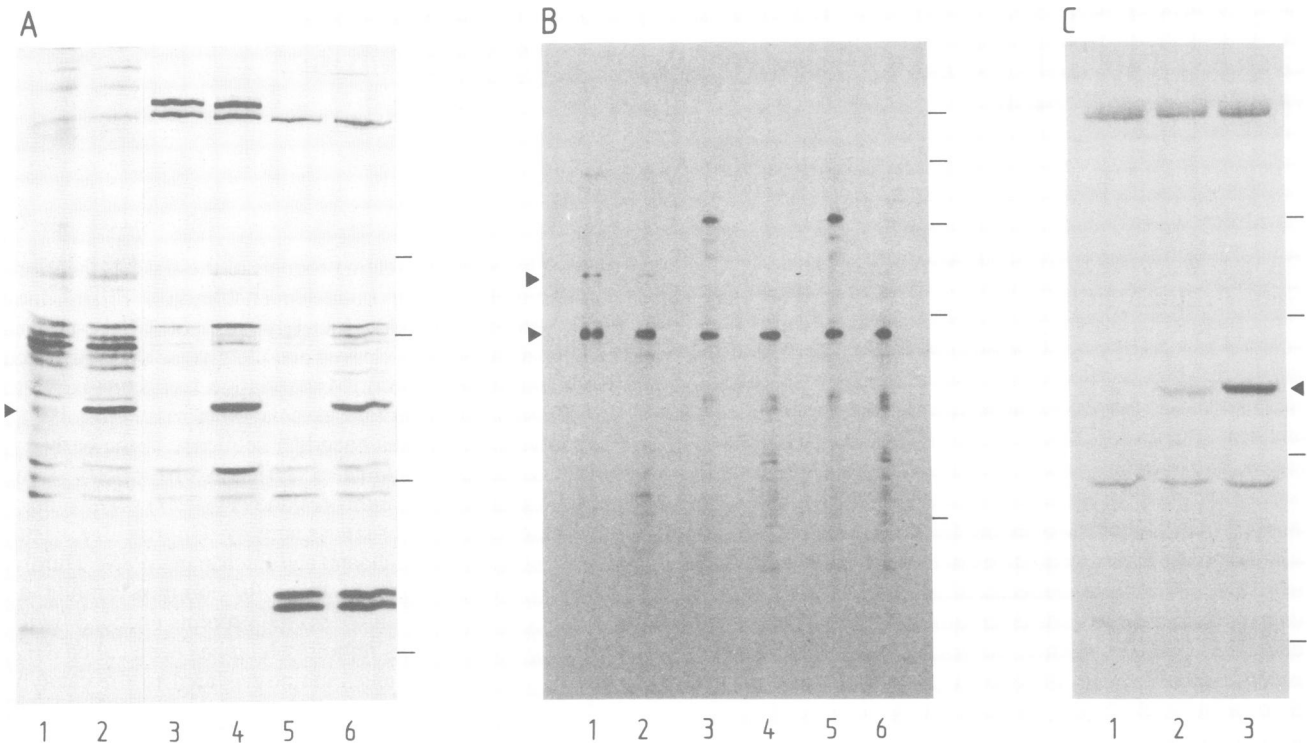

Fig. 1. Identification of the *fyn* gene product. **(A)** [^{35}S]methionine-labelled cell extracts from NIH-3T3 cells were immunoprecipitated with the following: no specific antibody (**lane 1**); mAb 327 against pp60$^{c\text{-}src}$ (**lane 2**); anti-fyn1 pre-incubated with excess fyn1 peptide (**lane 3**); anti-fyn1 (**lane 4**); anti-fyn2 pre-incubated with excess fyn2 peptide (**lane 5**); and anti-fyn2 (**lane 6**). The gel was treated with PPO/DMSO and exposed to Kodak XAR film for 2 days. The arrow shows the position of pp60$^{c\text{-}src}$. **(B)** The p59^{fyn} and pp60$^{c\text{-}src}$ bands from a gel similar to that in (A) were excised and digested with *S.aureus* V8 protease (20 ng for **lanes 1, 3** and **5**; 100 ng for **lanes 2, 4** and **6**). The figure shows the partial proteolytic maps for the proteins precipitated by mAb 327 (lanes 1 and 2); anti-fyn1 (lanes 3 and 4); and anti-fyn2 (lanes 5 and 6). Arrows indicate the positions of the two major proteolytic fragments of pp60$^{c\text{-}src}$. The gel was fluorographed and then exposed to film for 2 weeks. **(C)** [^{3}H]myristic acid-labelled cell extracts from NIH-3T3 cells were immunoprecipitated with the following: anti-fyn2 pre-incubated with excess fyn2 peptide (**lane 1**); anti-fyn2 (**lane 2**); and mAb 327 (**lane 3**). Exposure of fluorographed gels to preflashed film was for 3 weeks. The arrow shows the position of pp60$^{c\text{-}src}$. The positions of mol. wt standards are indicated at the right side of each figure and correspond (from top to bottom) to a mol. wt of 93, 67, 43 and 30 kd (and also 14 kd where applicable).

middle T antigen, the transforming protein of polyoma virus. As a result they have increased tyrosine kinase activity, which, at least in the case of pp60$^{c\text{-}src}$, appears to be necessary for transformation by middle T antigen (reviewed in Courtneidge, 1986). It is possible that another property which may be common to all *src* gene family members is complex formation with middle T antigen.

We are interested in defining the cellular function and regulation of *src* related proteins expressed in fibroblasts, and also in any part they might play in transformation by middle T antigen. One putative tyrosine kinase belonging to this family which has not been studied in any detail to date is *fyn*. The *fyn* gene (previously known both as *syn* and *slk*) was cloned by virtue of its homology to both v-*yes* (Nishizawa *et al.*, 1986; Semba *et al.*, 1986) and v-*fgr* sequences (Kawakami *et al.*, 1986). RNA analysis showed that *fyn* expression was broadly distributed, being present in most tissues examined although particularly high in the brain and absent in the kidney. Analysis of tissue culture lines showed that the protein product was likely to be expressed in fibroblasts (Semba *et al.*, 1986). We have generated anti-*fyn* specific antibodies, and in this report we describe their use in the characterization of the *fyn* encoded protein, as well as to study the regulation of its enzyme activity, and ability to bind to middle T antigen.

Results

Anti-fyn antibodies detect a 59 kd protein

The predicted protein sequence of *fyn* was examined to find unique antigenic peptide sequences. Because of the extreme similarity of members of the *src* family over most of their length (see Introduction), we concentrated our search on the first 80 amino acids of the sequence. Two peptides fulfilling our requirements were identified and synthesized (residues 22–35 called fyn1 and residues 35–51 called fyn2), coupled to a carrier protein and injected into rabbits. Antisera were tested by ELISA for reactivity against the peptides, and all antisera obtained after the 3rd week following the boost were found to be positive (data not shown).

The peptides we made corresponded to the predicted sequence of the human protein, and we therefore initially used human fibroblasts for our analyses. However, preliminary experiments indicated that the antibodies recognized the same protein in human and mouse cells (data not shown), and we will concentrate in this paper on data derived from NIH-3T3 cells and their derivatives. A protein that migrated electrophoretically slightly faster than pp60$^{c\text{-}src}$ (Figure 1A, lane 2) was immunoprecipitated from [^{35}S]methionine-labelled cells with antisera specific for fyn1 and fyn2 (Figure 1A, lanes 4 and 6). Preincubation of the

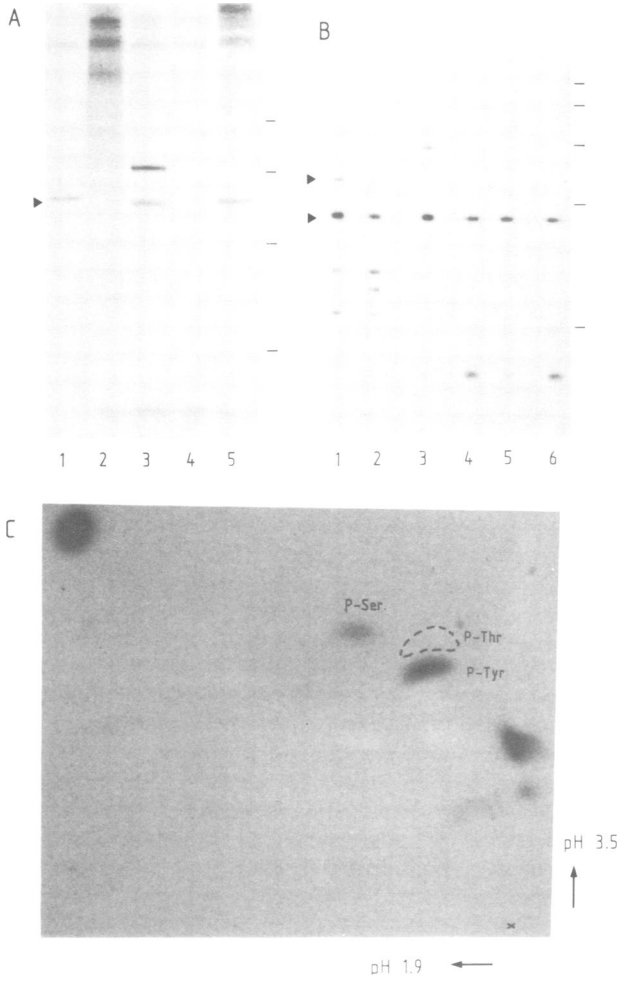

Fig. 2. Phosphorylation of the *fyn* gene product *in vivo*. **(A)** [^{32}P]orthophosphate labelled cell lysates were immunoprecipitated with the following: mAb 327 (**lane 1**); anti-fyn1 pre-incubated with excess fyn1 peptide (**lane 2**); anti-fyn1 (**lane 3**); anti-fyn2 pre-incubated with excess fyn2 peptide (**lane 4**); and anti-fyn2 (**lane 5**). The arrow shows the position of pp60$^{c\text{-}src}$. The gel was exposed to film for 24 h. **(B)** The bands from the gel shown in (A) were excised and digested with V8 protease (as indicated in Figure 1B). The figure shows the partial proteolytic maps for the proteins precipitated by mAb 327 (**lanes 1** and **2**); anti-fyn1 (**lanes 3** and **4**); and anti-fyn2 (**lanes 5** and **6**). The arrows denote the positions of the two major proteolytic fragments of pp60$^{c\text{-}src}$. Exposure time was 10 days. **(C)** A similar band to that in (A, lane 5) was excised from a gel and subjected to phosphoaminoacid analysis. The positions of unlabelled phosphoaminoacid standards are indicated. The origin is marked with an X. Exposure to preflashed film was for 4 weeks. The positions of mol. wt standards are indicated at the right side of each figure and correspond (from top to bottom) to a mol. wt of 93, 67, 43 and 30 kd (and also 14 kd where applicable).

antibodies with their specific peptides prevented immunoprecipitation of this protein (Figure 1A, lanes 3 and 5). There were no other immunoprecipitated proteins common to both antisera which could be blocked by pre-incubation with the relevant peptide. The relationship of these two 59 kd proteins to each other and to pp60$^{c\text{-}src}$ was investigated by excising the bands from the gel and subjecting them to partial V8 proteolysis (Figure 1B). The V8 proteolytic fragments of the 59 kd proteins immunoprecipitated by the two anti-peptide antibodies were identical (cf. lanes 3 and 4 with 5 and 6). Also, the fragments were similar to but distinct from those produced by V8 proteolysis of pp60$^{c\text{-}src}$ (lanes 1 and 2). For example one V8 fragment of the 59 kd protein co-migrated with the carboxyl-terminal V8 fragment of pp60$^{c\text{-}src}$.

We next determined whether the 59 kd protein immunoprecipitated by the anti-fyn antisera contained fatty acid. NIH-3T3 cells were labelled with [^{3}H]myristic acid and lysates were immunoprecipitated using anti-fyn antibodies. Anti-fyn2 antisera recognized a 59-kd myristylated protein (Figure 1C, lane 2). The precipitation of this protein was prevented by preincubation of antibody with fyn2 peptide (lane 1). As a positive control, we also show that mAb 327 against *src* proteins immunoprecipitated myristylated pp60$^{c\text{-}src}$ (lane 3). A 59 kd protein was also precipitated using anti-fyn1 antisera (data not shown).

We wished to examine the phosphorylation state of the 59 kd protein *in vivo*. Cell lysates from [^{32}P]orthophosphate-labelled NIH-3T3 cells were immunoprecipitated with anti-fyn1 and anti-fyn2 antibodies. A 59 kd phosphoprotein was detected by both antisera (Figure 2A, lanes 3 and 5). This protein was absent when the antisera were pre-incubated with the relevant peptides (lanes 2 and 4). Anti-fyn1 antibodies also immunoprecipitated a higher mol. wt phosphoprotein in this experiment; however this was not immunoprecipitated by anti-fyn2 antibodies. There were no other immunoprecipitated phosphoproteins common to both anti-fyn antisera. In order to analyse the 59 kd proteins further, the bands were excised from the gel and digested with V8 protease (Figure 2B). The V8 proteolytic fragments of the 59 kd proteins immunoprecipitated by the two anti-fyn antisera were identical (lanes 3 and 4 versus lanes 5 and 6). Also shown is a comparison between pp60$^{c\text{-}src}$ (from Figure 2A, lane 1) and the p59 proteins, demonstrating that the proteins are similar, but clearly distinct. To determine which amino acid residues were phosphorylated in the 59 kd protein, [^{32}P]orthophosphate-labelled 59 kd protein was immunoprecipitated using anti-fyn2 antibodies, excised from polyacrylamide gels and subjected to phosphoamino acid analysis. The results indicate that this protein was phosphorylated on serine and tyrosine residues *in vivo* (Figure 2C).

To summarize the *in vivo* labelling results, we have used anti-peptide antibodies to two sequences derived from the *fyn* gene to identify a 59 kd protein in NIH-3T3 cells. This protein, p59, was phosphorylated on serine and tyrosine residues and was also myristylated.

p59 has tyrosine kinase activity in vitro

The sequence of *fyn* has high homology with that of genes known to encode tyrosine kinases. Our next question therefore was whether our anti-fyn antibodies immunoprecipitated a tyrosine kinase activity. Immunoprecipitates were prepared using anti-fyn2 antisera and assayed for *in vitro* kinase activity under conditions used routinely to demonstrate pp60$^{c\text{-}src}$ kinase activity. We saw phosphorylation of p59 (Figure 3A, lane 3) which was prevented by preincubation of the antibody with fyn2 peptide (lane 2). The autophosphorylation of pp60$^{c\text{-}src}$ under the same conditions is shown in lane 1. The p59 band and pp60$^{c\text{-}src}$ were excised from the gel and digested with V8 protease (Figure 3B). The major labelled proteolytic fragment of the 59 kd protein co-migrated with the major pp60$^{c\text{-}src}$ fragment, however, there were minor differences. The same results have been obtained using anti-fyn1 antibodies (data not shown). The p59 band from similar experiments was subjected to

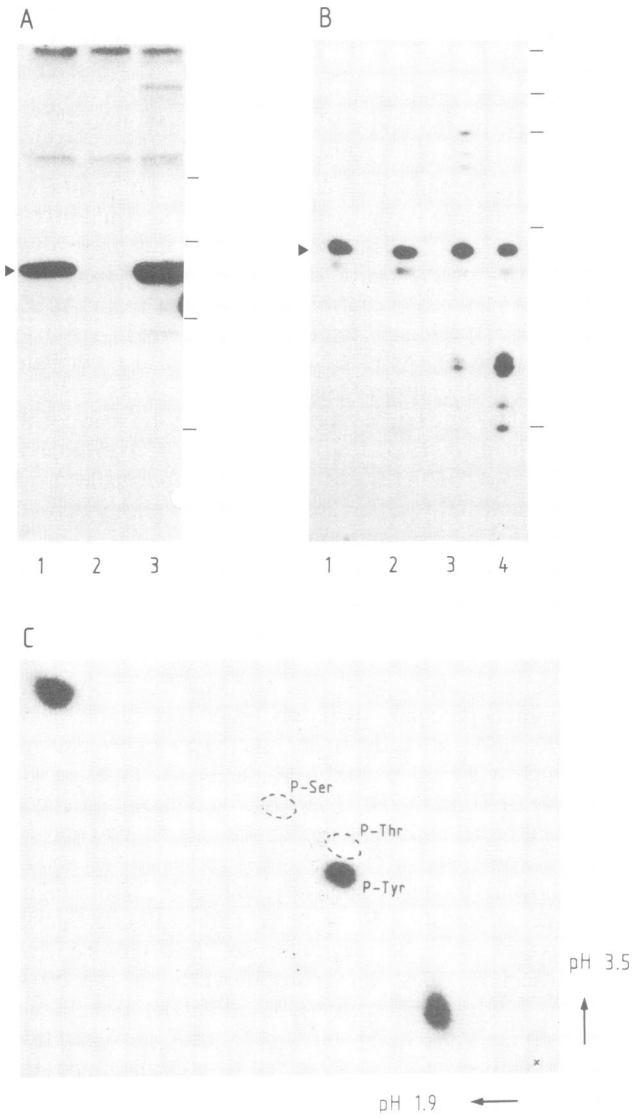

Fig. 3. *In vitro* tyrosine kinase activity in p59^{fyn} immunoprecipitates. **(A)** The immune complex kinase assay was carried out on NIH-3T3 cell lysates that had been immunoprecipitated with the following: mAb 327 (**lane 1**); anti-fyn2 pre-incubated with excess fyn2 peptide (**lane 2**); and anti-fyn2 (**lane 3**). The arrow shows the position of pp60$^{c\text{-}src}$. The gel was exposed to film overnight. **(B)** The bands from a gel similar to that in Figure 3A were excised and digested with V8 protease (as indicated in Figure 1B). The figure shows the partial proteolytic maps for the proteins precipitated by mAb 327 (**lanes 1** and **2**); and anti-fyn2 (**lanes 3** and **4**). The arrow indicates the position of the carboxy-terminal fragment of pp60$^{c\text{-}src}$. Exposure to film was for 10 days. **(C)** A similar band to that seen in (A, lane 3) was excised and subjected to two-dimensional phosphoaminoacid analysis. The positions of authentic standards are shown. The origin is marked with an X. Exposure to pre-flash film was for 14 days. The positions of mol. wt standards are indicated at the right side of each figure and correspond (from top to bottom) to a mol. wt of 93, 67, 43 and 30 kd (and also 14 kd where applicable).

phosphoamino acid analysis and the results indicated that phosphorylation of p59 was exclusively on tyrosine residues (Figure 3C). In order to ask whether the *in vitro* kinase activity was intrinsic to the immunoprecipitated p59, experiments using lysates fractionated on sucrose density gradients were conducted. Fractions were collected from the gradient, immunoprecipitated with anti-fyn2 antisera and tested for tyrosine kinase activity. There was a single peak

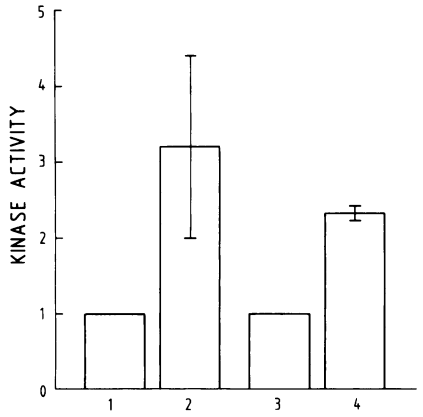

Fig. 4. Effect of dephosphorylation on p59^{fyn} tyrosine kinase activity. Lysates were prepared from NIH-3T3 cells either in the presence (**lanes 1** and **3**) or in the absence (**lanes 2** and **4**) of 100 μM sodium orthovanadate, pp60$^{c\text{-}src}$ and p59^{fyn} were immunoprecipitated from these lysates and assayed for kinase activity by measuring the number of counts incorporated into enolase. Depicted is the relative kinase activity (the activity for each enzyme in the presence of vanadate has been set at 1). The reactions were carried out in duplicate and error bars show the range of values obtained.

of tyrosine kinase activity in the fractions corresponding to a mol. wt of ~60 kd (data not shown), suggesting that the kinase activity was due to autophosphorylation of monomeric p59.

All the data presented thus far strongly suggest that we have identified the protein product predicted from the *fyn* gene sequence. Therefore the 59 kd protein will now be referred to as p59^{fyn}.

p59^{fyn} kinase activity is regulated by tyrosine phosphorylation

The tyrosine kinase activity of pp60$^{c\text{-}src}$ is negatively regulated by tyrosine phosphorylation at its major tyrosine phosphorylation site, Tyr-527. Since the predicted sequence of p59^{fyn} contains a tyrosine residue at a corresponding site (Tyr-531), it may also be subject to similar regulation. In order to investigate this possibility the effects of tyrosine dephosphorylation of p59^{fyn} kinase activity were examined. The extent of tyrosine phosphorylation of proteins in cell lysates can be conveniently manipulated using the specific phosphotyrosine phosphatase inhibitor, sodium orthovanadate (Courtneidge, 1985). We therefore prepared lysates in the presence and absence of sodium orthovanadate, immunoprecipitated p59^{fyn} and pp60$^{c\text{-}src}$ and measured their tyrosine kinase activities. Consistent with previous reports, the kinase activity of pp60$^{c\text{-}src}$ from lysates prepared without the inhibitor was ~3-fold higher than that in lysates containing sodium orthovanadate (Figure 4, lanes 1 and 2). The kinase activity of p59^{fyn} was affected similarly, being 2-fold higher in lysates prepared without sodium orthovanadate (Figure 4, lanes 3 and 4), strongly suggesting that the *fyn* protein is also regulated by phosphorylation at tyrosine.

p59^{fyn} forms a complex with polyoma virus middle T antigen

It has previously been reported that middle T antigen, the transforming protein of polyoma virus, is complexed to both pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983, 1984) and pp62$^{c\text{-}yes}$ (Kornbluth *et al.*, 1987). Since *fyn* is also a member of the

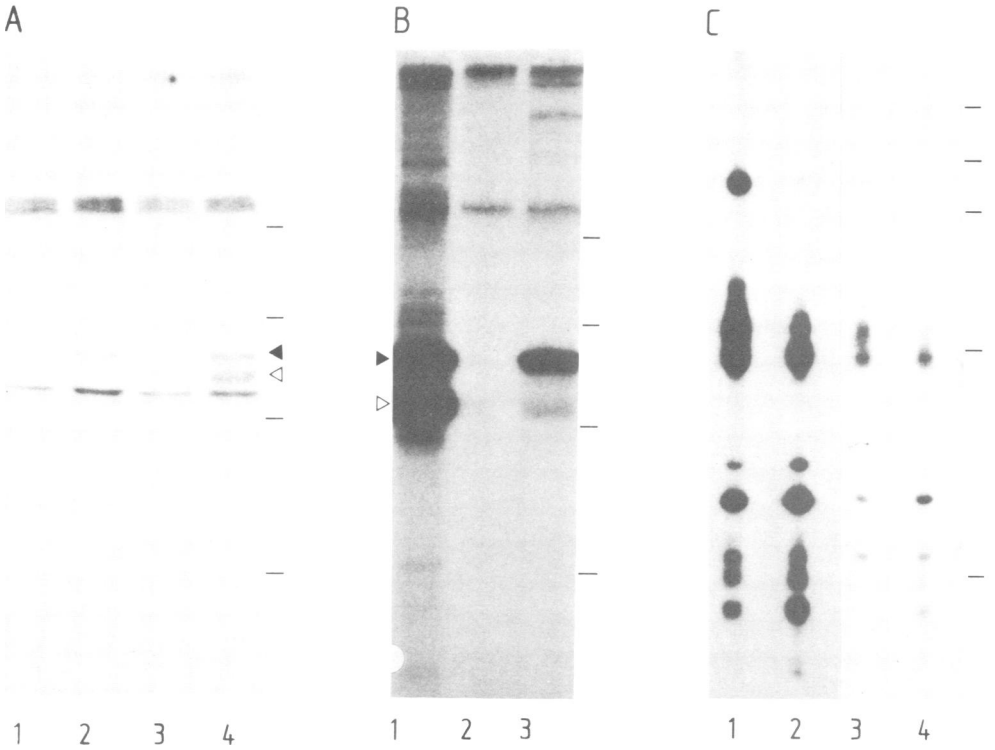

Fig. 5. p59^{fyn} forms a complex with polyoma virus middle T antigen. (**A**) Cell extracts from normal (**lanes 1** and **2**) and middle T transformed (**lanes 3** and **4**) NIH-3T3 cells were immunoprecipitated as follows: anti-fyn2 pre-incubated with excess fyn2 peptide (lanes 1 and 3); and anti-fyn2 (**lanes 2** and **4**). The filled arrow indicates the position of p59^{fyn} and the open arrow indicates the position of middle T antigen. The gel was treated with 1 M potassium hydroxide at 55°C for 1 h, stained, dried and exposed to film for 5 days. (**B**) Cell extracts from dl8 middle T transformed NIH-3T3 cells were immunoprecipitated using the following: mAb 327 against pp60$^{c\text{-}src}$ (**lane 1**); anti-fyn2 pre-incubated with excess fyn2 peptide (**lane 2**); and anti-fyn2 (**lane 3**). The filled arrow indicates the position of pp60$^{c\text{-}src}$ and the open arrow indicates the position of dl8 middle T antigen. The gel was exposed to film for 2 days. (**C**) Authentic dl8 middle T (B, lane 1) and the co-migrating band in (B, lane 3) were excised and digested with V8 protease (see legend to Figure 1B for details). **Lanes 1** and **2** are the proteolytic fragments of authentic dl8 middle T antigen phosphorylated by pp60$^{c\text{-}src}$, **lanes 3** and **4** are the proteolytic fragments of the 55 kd protein phosphorylated by p59^{fyn}. Exposure to film was for 15 h (lanes 1 and 2) and 7 days (lanes 3 and 4). The positions of mol. wt standards are indicated at the right side of each figure and correspond (from top to bottom) to a mol. wt of 93, 67, 43 and 30 kd (and also 14 kd where applicable).

src family and expressed in fibroblasts we wished to find out whether middle T antigen also complexed to p59^{fyn}. We therefore immunoprecipitated p59^{fyn} from lysates of normal and middle T transformed NIH-3T3 cells and performed kinase assays. As shown earlier, a single band of autophosphorylation p59^{fyn} was seen in the normal cells (Figure 5A, lane 2). In contrast, kinase assays from the transformed cells showed both p59^{fyn} and a weakly phosphorylated band which had the mobility of middle T antigen (lane 4). These bands were not present when the antisera were preincubated with fyn peptide (lanes 1 and 3). While these data suggest that p59^{fyn} can indeed associate with middle T antigen, the amount of complex detected appeared very low. Therefore the relative amounts of pp60$^{c\text{-}src}$:middle T and p59^{fyn}:middle T complexes were compared by kinase assay (Figure 5B). Here we used cells transformed with the truncated middle T mutant, dl8, since this allowed better resolution of the complexed proteins on gels. The complex of pp60$^{c\text{-}src}$ and dl8 middle T antigen is shown in lane 1. The *fyn* immunoprecipitate, assayed under identical conditions, is shown in lane 3; a band which co-migrates with middle T antigen is just detectable. We confirmed that this band was indeed middle T antigen by digestion with V8 protease and comparison with V8 proteolytic fragments from authentic dl8 middle T antigen (Figure 5C). However, we estimate that the extent of middle T phosphorylation in pp60$^{c\text{-}src}$ complexes is ≥ 10-fold higher than that in p59^{fyn} complexes.

Table I. The association of mutant middle T antigens with p59^{fyn} and pp60$^{c\text{-}src}$

Mutant class	Example	Association with pp60$^{c\text{-}src}$	Association with p59^{fyn}
I	Wild-type	+	+
	dl8	+	+
II	dl23	+	+
	dl1015	+	+
III	NG59	−	−

Note that class I middle T mutants are transformation positive, class II and class III middle T mutants are transformation negative (Courtneidge and Heber, 1987).

There are many mutants of middle T antigen, and we used a panel of 3T3 cell lines that had been transfected with some of these mutants to examine middle T binding to p59^{fyn} in more detail. The results are shown in Table I. Whereas p59^{fyn} was able to bind to both transforming (wild-type and dl8) and two non-transforming (dl23 and dl1015) middle T antigens, it did not appear to associate with the non-transforming NG59 middle T. In this respect, it resembled pp60$^{c\text{-}src}$. However, in all cases where any association was

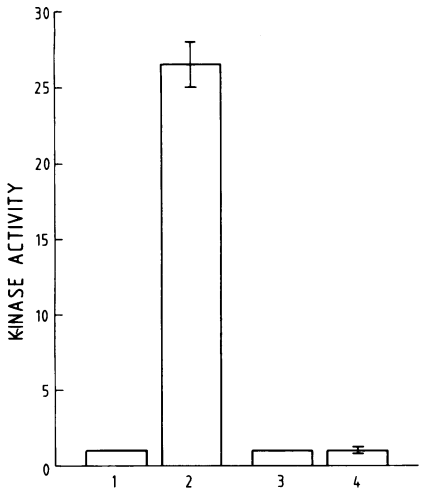

Fig. 6. Comparison of p59^{fyn} and pp60$^{c\text{-}src}$ tyrosine kinase activities in normal and middle T transformed fibroblasts. pp60$^{c\text{-}src}$ and p59^{fyn} were immunoprecipitated from lysates of normal and middle T transformed NIH-3T3 cells and assayed for kinase activity using enolase as substrate. Depicted is the relative kinase activity (the activity in normal cells has been set at 1 for each enzyme). The reactions were carried out in duplicate and error bars show the range of values obtained.

seen, it was present at $<10\%$ of the level of the pp60$^{c\text{-}src}$:middle T complexes (as judged by kinase assay).

To determine whether p59^{fyn} was activated in middle T transformed cells we compared the *in vitro* kinase activities of p59^{fyn} in normal versus transformed NIH-3T3 cells. The kinase activity was quantitated by measuring the counts incorporated into an exogenous substrate, acid-denatured enolase as previously described (Courtneidge, 1985). In control experiments pp60$^{c\text{-}src}$ kinase activity was elevated ~27-fold in middle T-transformed cells when compared with normal cells (Figure 6, lane 1 versus lane 2). In contrast, p59^{fyn} kinase activity was the same in both cell lines (lane 3 versus lane 4).

Discussion

Identification of the fyn gene product

In this study we have identified the gene product of *fyn* in normal cells using antibodies to unique peptides as deduced from the *fyn* cDNA sequence. Several lines of evidence lead us to conclude that the p59 we have identified is the *fyn* gene product. Firstly, two different peptides were synthesized whose sequences were chosen from the unique amino-terminal domain of the *fyn* encoded protein. Antisera to both peptides immunoprecipitated the same 59 kd protein. Secondly, p59 was labelled with both [^{32}P]orthophosphate and by [^{3}H]myristic acid as would be predicted for a *src* family member. Thirdly, immunoprecipitates containing p59 had intrinsic tyrosine kinase activity *in vitro*. These experiments have all been repeated using affinity-purified anti-peptide antibodies and the results obtained were essentially the same. These results are consistent with a recent report by Kawakami *et al.* (1988) who identified a 59 kd *fyn*-encoded protein in over-producing transfected cell lines.

The partial proteolytic map for p59^{fyn} not only showed that both anti-fyn peptide antibodies were immunoprecipitating the same protein but also showed differences and similarities with the V8 map for pp60$^{c\text{-}src}$. For example one V8 fragment of p59^{fyn} co-migrated with the carboxy-terminal V8 fragment of pp60$^{c\text{-}src}$, and given the sequence similarities between these two proteins in their carboxy-terminal domains, this fragment may represent the equivalent domain of the *fyn* protein. Other fragments however appeared distinct, and may represent cuts within the unique amino terminus of the *fyn* protein.

We also showed here that p59^{fyn} had covalently attached myristic acid. In pp60$^{c\text{-}src}$, the amino-terminal residues which have been shown to be critical for myristylation of pp60$^{c\text{-}src}$ are Gly-2 (which is myristylated) and Lys-7. Interestingly, Gly-2 and Lys-7 are the only residues that pp60$^{c\text{-}src}$ and p59^{fyn} have in common at their amino-termini, in keeping with the notion that they are sufficient to act as the myristylation signal in the right context (Kaplan *et al.*, 1988).

We showed that p59^{fyn} was phosphorylated both *in vivo* and *in vitro* on tyrosine residues. pp60$^{c\text{-}src}$ is phosphorylated *in vivo* at Tyr-527 (Cooper *et al.*, 1986) and *in vitro* at Tyr-416 (Smart *et al.*, 1981). In p59^{fyn} Tyr-531 is the positional equivalent of pp60$^{c\text{-}src}$ Tyr-527, and Tyr-420 the putative autophosphorylation site. Consistent with this, V8 analysis of p59^{fyn} labelled with ^{32}P *in vivo* and auto-phosphorylated *in vitro* showed that the presumed carboxy-terminal fragment was labelled in both cases. However, p59^{fyn} labelled *in vitro* had other phosphorylated fragments, perhaps indicating a potential for autophosphorylation at other tyrosine sites in this protein.

Our analysis of [^{32}P]orthophosphate-labelled cells showed that p59^{fyn} was also phosphorylated on serine residues *in vivo* (and we do not as yet rule out the possibility that it also contains a low level of phosphothreonine). pp60$^{c\text{-}src}$ is phosphorylated at Ser-17, which has been shown to be a substrate for cAMP-dependent protein kinase (Roth *et al.*, 1983). However, this site is not present in p59^{fyn} and there are no serines (or threonines) that lie within the canonical phosphorylation sequence for cAMP-dependent protein kinase (for references see Edelman *et al.*, 1987). There are however a number of serine and threonine residues that are potential substrates for protein kinase C, calmodulin-dependent protein kinase and the casein kinases. It remains to be seen which kinases phosphorylate p59^{fyn} and what effect these phosphorylation(s) have on p59^{fyn} kinase activity. We are currently generating cell lines which over-express the *fyn* protein, and intend to use these to map the phosphorylation sites as a prelude to answering these questions.

Regulation of p59^{fyn} tyrosine activity by tyrosine phosphorylation

We have shown (both by autophosphorylation and by phosphorylation of exogenous substrates) that p59^{fyn} has tyrosine kinase activity *in vitro*. Here we showed that p59^{fyn}, like pp60$^{c\text{-}src}$, was negatively regulated by tyrosine phosphorylation. However, the extent to which p59^{fyn} was activated was never as high as that for pp60$^{c\text{-}src}$, and appeared to be more dependent upon cell confluency (unpublished results). It may be that p59^{fyn} is already in a more active state than pp60$^{c\text{-}src}$ in NIH-3T3 cells or that there are both positive and negative regulatory tyrosine sites in p59^{fyn}.

By analogy with the negative regulatory phosphorylation site of pp60$^{c\text{-}src}$ (Tyr-527), Tyr-531 of p59^{fyn} may be the site

at which phosphatases act to elevate its kinase activity *in vitro*. When this site is mutated to Phe in pp60$^{c\text{-}src}$ the tyrosine kinase activity is elevated between 5- and 13-fold and the mutant protein is oncogenic (Cartwright *et al.*, 1987; Kmiecik *et al.*, 1987; Piwnica-Worms *et al.*, 1987). This result has also been shown for the equivalent tyrosine site in the *lck* protein (Amrein and Sefton, 1988; Marth *et al.*, 1988). The oncogenic potential of p59^{fyn} has been implicated by construction of a fusion protein encoded by 5′ sequences for v-*fgr* (the oncogene present in Gardner-Rasheed feline sarcoma virus) and the 3′ two-thirds of the *fyn* gene. This chimeric protein transformed cells (Kawakami *et al.*, 1986). However it is not easy to assess the relative contributions of the *fyn* and *fgr* sequences to the oncogenic process. It is interesting that the carboxy terminal sequences of *fyn*, including Tyr-531, were present in the fusion protein. In contrast, mutant *fyn* proteins have recently been described, which appear to have sustained deletions in their carboxy termini, and which are transforming (Kawakami *et al.*, 1988).

p59^{fyn} complexes polyoma virus middle T antigen

We show here that p59^{fyn}, the third member of the *src* family whose gene is known to be expressed in fibroblasts, like pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$, could also form a complex with middle T antigen. However, the amount of complex appeared to be very low when compared to the middle T antigen:pp60$^{c\text{-}src}$ complex, and indeed could only be reproducibly detected after potassium hydroxide treatment of the gels to enhance the detection of phosphotyrosine. The middle T antigen:pp60$^{c\text{-}src}$ complex contains a third protein, p81, which may be a phosphatidylinositol kinase (Courtneidge and Heber, 1987). We were unable to detect this protein in the p59^{fyn}:middle T complex, but because of the low levels of the complex, we cannot exclude its presence. Indeed, the complexed form of p59^{fyn} sedimented at about the same region on sucrose density gradients (200−220 kd) as the complexed form of pp60$^{c\text{-}src}$, suggesting that another protein may be present (unpublished observations). We have examined a number of middle T mutants and shown that those which bind pp60$^{c\text{-}src}$ also bind p59^{fyn} (presumably in separate complexes), but once again the amount of middle T binding to the *fyn* protein was very much lower in each case. Many mutants of middle T antigen (those in class II) bind to pp60$^{c\text{-}src}$ yet are non-transforming. We show here that these mutants also bound to p59^{fyn}. The transformation defect of these mutants is therefore still unresolved.

In order to see whether p59^{fyn} was activated in middle T transformed cells we compared the enzyme activity from normal and transformed cells. Our results showed that whereas pp60$^{c\text{-}src}$ kinase activity was elevated considerably in the transformed cells, p59^{fyn} kinase activity was unchanged. There are a number of possible explanations for these results. First, the antibodies may not be capable of recognizing the complexed form of p59^{fyn} very well. We do not believe this to be the case since antibodies to similar amino-terminal regions of pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ recognize their respective proteins complexed with middle T antigen (Parsons *et al.*, 1986; Kornbluth *et al.*, 1987; Cheng *et al.*, 1988), and the carboxy terminus of pp60$^{c\text{-}src}$ has been implicated in middle T binding (Cheng *et al.*, 1988). Second, it may be that there is as much p59^{fyn} as pp60$^{c\text{-}src}$ complexed to middle T antigen, but the complexed form of p59^{fyn} is not activated. Third, the complexed form of p59^{fyn} may be much less abundant than that of pp60$^{c\text{-}src}$, and any activation would therefore be obscured by the non-complexed form of p59^{fyn}. We have carried out preliminary experiments in order to try and distinguish between the second and third possibilities and our data suggest that in fibroblasts transformed by middle T there is very little p59^{fyn}:middle T complex. While we interpret these data to mean that p59^{fyn} binding to middle T is unlikely to play a major role in transformation of fibroblasts *in vitro*, we cannot rule out that, for example, a change in substrate specificity of p59^{fyn} caused by middle T binding would be important. In addition, in particular tissues or at particular times in development p59^{fyn} levels may be such that this protein would become a crucial target for middle T antigen *in vivo*.

Materials and methods

Cells and tissues

Cells used where NIH 3T3 fibroblasts and their derivatives which contain various middle T antigens, described in Courtneidge and Heber (1987) and in Cheng *et al.* (1986).

Antibodies

pp60$^{c\text{-}src}$ was immunoprecipitated using mAb 327 which is specific for *src* protein (Lipsich *et al.*, 1983). Middle T antigen was immunoprecipitated using serum from a rat bearing tumours induced by polyoma virus (RK serum; Courtneidge and Smith, 1983).

Generation of antibodies to the *fyn* encoded protein was as follows: peptides corresponding to amino acid residues 22−35 (fyn1: LNQSSGYRYGTDPT) and 35−51 (fyn2: TPQHYPSFGVTSIPNYN) were synthesized by Fmoc chemistry. The peptides were coupled to carrier as follows: equal weights of peptide and Keyhole limpet hemocyanin (Calbiochem) were dissolved in 0.1M $NaHCO_3$ at a concentration of 2 carrier mg/ml. Glutaraldehyde (Sigma) was added to peptide and carrier solution (pH 8.4) to a final concentration of 0.05% (v/v), and mixed at room temperature overnight. Glycine ethyl ester (pH 8.0) was added to a final concentration of 0.1 M and left for 30 min at room temperature. The coupled carrier was then precipitated with acetone, pelleted and redispersed in 0.9% NaCl at 1 mg carrier/ml. The conjugates were emulsified with Freund's complete adjuvant and 200 μg was injected into the lymph nodes of rabbits. After 1 week 200 μg of the conjugates in Freund's incomplete adjuvant were injected i.d. and 2 weeks later 500 μg of the conjugates in Freund's incomplete adjuvant were injected i.p. Further booster injections with 50 μg of the conjugates were introduced i.m. The rabbit sera were tested for the presence of anti-peptide antibodies by enzyme-linked immunoassay (Evan, 1985). Positive samples were then further tested for their ability to precipitate proteins from radiolabelled cell extracts.

The antisera were affinity-purified as follows: the immunoglobulins were precipitated with sodium sulphate (0.18 g/ml antiserum) for 15 min at 30°C, collected by centrifugation, redissolved in phosphate-buffered saline (PBS) and dialysed in PBS overnight. Affinity-purification was carried out using a column containing the relevant peptide coupled to CNBr-activated Sepharose 4B (as described in *Affinity Chromatography* by Pharmacia). Briefly, the antibodies were recycled through the column overnight, the column was washed with PBS followed by 2 M KCl and then the antibodies were eluted with 1 M propionic acid and immediately neutralized with 2 M potassium hydrogen phosphate. The peak fractions as measured by protein concentration were pooled and dialysed against three changes of PBS.

General methods

Our methods for labelling of cells, immunoprecipitation of proteins, kinase assays, sucrose density gradients, SDS−PAGE and peptide mapping have all been described before (Courtneidge and Smith, 1983, 1984; Courtneidge *et al.*, 1984; Courtneidge, 1985) but some changes have been introduced and are described below. Phosphoamino acid analysis was by the method described in Cooper *et al.* (1983).

In vivo labelling

Cells were grown to confluency in 10-cm dishes and then incubated in either methionine-free DMEM for 2 h followed by addition of 250 μCi/ml of [^{35}S]methionine (Amersham) for 4−6 h, or in phosphate-free DMEM for

3 h followed by addition of 500 μCi/ml of carrier-free [^{32}P]orthophosphate (Amersham) for 3–4 h. Myristate labelling was for 18 h in DMEM containing 0.5% fetal calf serum, 1 mM pyruvate and 100 μCi/ml of [9,10(*N*)-^{3}H] myristic acid (Amersham).

Preparation of lysates, immunoprecipitations and kinase assays

In general, lysates were prepared using RIPA lysis buffer, which is RIPA buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 μM sodium orthovanadate] containing 1% Trasylol and 20 μM leupeptin. Cells were washed twice in TBS (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT, 100 μM sodium orthovanadate) and then scraped from the dish in 0.5 ml RIPA lysis buffer. The lysed cells were transferred to cold microfuge tubes which were vortexed and incubated on ice for 10 min and then centrifuged for 10 min to remove nuclei and cell debris [at this stage *in vivo* labelled extracts were incubated with *Staphylococcus aureus* (to 10%) for 30 min at 4°C, and the latter was then removed by centrifugation]. The supernatants were incubated with antibody for 90 min at 4°C. In the negative controls the antibodies were premixed with excess specific peptide for 5 min (3 mg/ml peptide added in an equal volume to that of the antibody). Immune complexes were then collected by incubation with *S.aureus* for 30 min at 4°C (the *S.aureus* used with the *in vivo* labelled extracts had been preincubated with unlabelled cell lysate for 30 min and then washed 3 times in RIPA buffer). The immunoprecipitates were washed 3 times in RIPA buffer and once in TBS. For kinase assays the immunoprecipitates were resuspended in 20 μl of kinase buffer (Courtneidge, 1985) containing 2 μCi[γ-^{32}P]ATP and incubated at 30°C for 4 min. SDS sample buffer was used to terminate the reactions.

In the experiments designed to study the effects of phosphotyrosine phosphatases, the cells were scraped into TBS (with or without vanadate), pelleted at 1500 r.p.m. for 5 min at 4°C and then the cell pellet was resuspended in lysis buffer containing NP-40 (Courtneidge, 1985). The preparation of the sodium orthovanadate (Brown and Gordon, 1984) is important and the method is therefore reiterated here. Sodium orthovanadate is dissolved in double-distilled water to give an ~10× solution. The solution is adjusted to pH 10 (at which point it turns yellow) and then boiled until colourless. It is then cooled to room temperature, reset to pH 10, and boiled again. This cycle is repeated until the pH remains at 10 after boiling (usually 5 times). The volume is adjusted and the solution is stored at 4°C for up to 2 weeks.

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